AL/EQ-TR-1993-0026




# BIOLOGICAL DEGRADATION OF TETRACHLOROETHYLENE IN METHANOGENIC CONDITIONS

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June 1994

Final Technical Report for Period July 1991 - January 1993

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ARMSTRONG LABORATORY



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# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | June 1994 | Final 12 Jul 91 - 11 Jan 93 |

**4. TITLE AND SUBTITLE**

Biological Degradation of Tetrachloroethylene in Methanogenic Conditions

**5. FUNDING NUMBERS**

C:F08635-86-C-0181

**6. AUTHOR(S)**

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**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

AL/EQW
139 Barnes Drive, Suite 2
Tyndall AFB FL 32403-5319

**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**

AL/EQ-TR-1993-0026

**11. SUPPLEMENTARY NOTES**

Availability of This Report Is Specified on Reverse of Front Cover
Cathy Vogel AL/EQW-OL Comm 904-283-6035 AUTOVON 523-6035

**12a. DISTRIBUTION/AVAILABILITY STATEMENT**

Approved For Public Release
Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** (Maximum 200 words)

Research objective: investigate anaerobic biodegradation of perchloroethylene (PCE). Specific objectives: (1) determine if the presence of PCE is necessary to sustain dechlorination of vinyl chloride (VC), (2) delineate the role of hydrogen ($H_2$) in PCE reductive dechlorination, (3) investigate the ability of the high-level PCE/methanol (MeOH) culture to utilize low levels of PCE, and (4) determine the applicability of an Anaerobic Attached-film Expanded-bed (AAFEB) reactor to achieve PCE dechlorination. The investigators determined: (1) by using a VC-fed culture unable to sustain ETH production, that the presence of PCE is required to sustain VC dechlorination, (2) $H_2$ acts as the electron donor directly used for the reductive dechlorination of PCE to ethene, (3) the PCE/MeOH culture was able to use ppb levels of PCE due to the small requirement for electron donor ($H_2$) by the culture, and (4) that the loss of the dechlorinating biomass from the suport matrix, and/or the inability of the culture to support PCE dechlorination at low concentrations, led to the failure of the AAFEB reactor system.

**14. SUBJECT TERMS**

Biodegradation, Tetrachloroethylene, Methanogenesis, Fixed-film Reactors, Biological Treatment, Chlorinated Hydrocarbons

**15. NUMBER OF PAGES**

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | UL |



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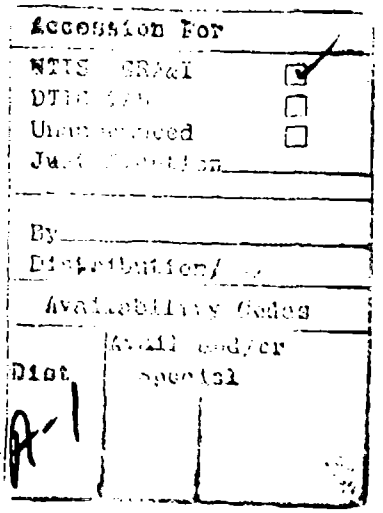
## PREFACE

This report was prepared by James M. Gossett, Cornell University, School of Civil and Environmental Engineering, Hollister Hall, Ithaca, NY 14853-3501, under Contract Number F08635-91-C-0181 for the US Air Force Civil Engineering Support Agency (AFCESA/EQW), Tyndall AFB FL 32403-5319.

This report summarizes work completed between 12 Jul 91 and 11 Jan 93. The HQ AFCESA/EQW project officer was Catherine M. Vogel.



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# EXECUTIVE SUMMARY

## A. OBJECTIVE

Tetrachloroethene (also known as perchloroethylene, or PCE) and the less-chlorinated ethenes produced from it via reductive dehalogenation — trichloroethene (TCE), dichloroethene (DCE) isomers and vinyl chloride (VC) — have become common groundwater pollutants throughout the United States, including USAF IRP sites. PCE can be reductively dechlorinated to ethene (ETH) by anaerobic microorganisms; however, the promise of anaerobic bioremediation for chlorinated ethenes has not been realized, largely due to a lack of understanding of microbiological fundamentals.

Specific objectives of this research were:

- To determine if PCE's presence is necessary to sustain dechlorination of VC;
- To delineate the role of hydrogen ($H_2$) in reductive dechlorination of PCE;
- To investigate the ability of our high-level PCE/methanol (MeOH) culture to utilize low levels of PCE — a critical factor in determining success or failure of our proposed bioremediation system;
- To investigate the potential of an Anaerobic Attached-Film Expanded-Bed (AAFEB) reactor to achieve continuous-flow dechlorination of PCE.

## B. BACKGROUND

Under anaerobic conditions, PCE can be completely dechlorinated to ETH by a stepwise, reductive dehalogenation process; however, the final reduction step is apparently rate-limiting, with significant levels of the intermediate VC typically observed. Our first experimental objective derives from our repeated observation that VC dechlorination and ETH production are readily sustained in PCE-fed systems, whereas we have experienced difficulty with VC as the sole chlorinated specie. PCE's presence may be necessary to induce a relatively nonspecific dechlorination activity capable of acting upon many other chlorinated species — including VC.

Dechlorination via a reductive mechanism requires an electron donor. Since several alternate donors appear to serve the dechlorination process, our hypothesis is that the simplest — $H_2$ — may be the donor directly involved. This hypothesis was addressed in our second experimental objective.

In recent, unsponsored studies, we developed an anaerobic MeOH/PCE enrichment culture which dechlorinates PCE at unprecedented, high rates with efficient use of MeOH as the electron donor for reductive dechlorination. The MeOH/PCE culture produced little or no methane ($CH_4$) when high PCE doses were added, presumably because of inhibition of methanogenesis by PCE.

What remains to be determined is whether the subsequent use of this high-level culture at lower, noninhibitory concentrations of PCE will spark a rise in methanogenic activity, consuming an ever-increasing fraction of MeOH equivalents, necessitating ever-greater additions of MeOH to allow sustenance of dechlorination. These issues were addressed in our third experimental objective.

Our earlier efforts to achieve continuous-flow dechlorination of PCE met with only limited success; quantitative conversion of PCE to VC resulted, with only minor amounts of ETH produced. The AAFEB reactor offers essentially the complete-mix conditions of a suspended-growth system (i.e., a system where we *have* met with significant success), while providing a mean-cell residence time (or "solids retention time", SRT) greatly in excess of the hydraulic retention time (HRT). The AAFEB system was evaluated as part of our fourth experimental objective.

## C. SCOPE

Early experiments concerning the sustenance of dechlorination of VC in the absence of PCE were completed using a methanogenic culture which had demonstrated capability in dechlorinating PCE at low concentrations (0.5 mg/L) to VC and ETH. Experiments were completed at 35°C, in either batch or semicontinuous mode with the suspended mixed culture in 160-mL serum bottles. Batch doses of PCE and/or VC were delivered to the cultures and the resultant transformations were monitored using a headspace gas chromatographic technique. In some studies, repetitive additions of PCE and/or VC were administered on a regular basis, over many months — along with MeOH which served as electron donor. The resultant ETH productions of VC-fed systems were compared with those of PCE-fed cultures to determine the need for PCE to sustain complete dechlorination.

Increasing doses of PCE were given to selected cultures to encourage development of a culture in which dechlorination was a major activity. An anaerobic culture (termed "high-PCE/MeOH") with superior PCE reduction capability was developed from this action. Selective inhibitors were applied to this culture to elucidate the role of the PCE dechlorinating organism(s) of this culture. $H_2$ and MeOH were employed as electron donors during inhibitor studies to facilitate a more thorough understanding of the roles of major classes of organisms, and to delineate the role of $H_2$ in dechlorination.

The ability of the high-PCE/MeOH culture to sustain dechlorination at low PCE levels was investigated in semicontinuous, serum-bottle studies at 35°C. Two different levels of PCE and three different MeOH:PCE ratios were employed. Over a 34- to 42-day period, the patterns of VC and ETH production following repeated additions of PCE and MeOH were observed and compared.

An AAFEB reactor was designed, constructed and operated over a 200-day period at 35°C. The liquid volume was 1.56 liters; the bed media consisted of 100-μm diatomaceous earth particles. PCE dissolved in a prereduced basal medium was continuously pumped to the reactor from a feed reservoir; a concentrated MeOH/water mixture was continuously injected directly into the bed via a syringe pump. Performance was monitored by headspace gas chromatography and by removal of liquid samples for analysis of nonvolatiles. Several different influent PCE concentrations, MeOH:PCE ratios, and HRTs were explored.

## D. RESULTS

Fairly constant conversion of PCE to ETH was sustainable by a MeOH-enriched, PCE-fed culture. When VC additions were substituted for PCE, an ever-decreasing ETH production rate was observed. The decrease in ETH-production rate could be somewhat reversed if PCE additions were resumed.

$H_2$ served as electron donor in the reductive dechlorination of PCE to VC and ETH for periods up to 40 days in anaerobic enrichment cultures; however, sustained dechlorination for more extended periods required the addition of filtered supernatant from a MeOH-fed culture. This suggests a nutritional dependency of $H_2$-using dechlorinators upon the metabolic products of other organisms in the more diverse, MeOH-fed system. Vancomycin, an inhibitor of cell-wall synthesis in eubacteria, was found to inhibit acetogenesis when added at 100 mg/liter to both MeOH-fed and $H_2$-fed cultures. The effect of vancomycin on dechlorination was more complex. MeOH could not sustain dechlorination when vancomycin inhibited acetogenesis, while $H_2$ could.

With repetitive additions of MeOH and PCE at a 2:1 ratio (eq/eq), using noninhibitory levels of PCE (ca. 9 mg/L), methanogenesis consumed an ever-increasing fraction of MeOH equivalents, decreasing the extent of dechlorination. Dechlorination of PCE at such concentrations was sustained only if higher ratios of MeOH:PCE were employed. However, even then, the production of ETH gradually decreased as an increasing fraction of the added PCE remained as VC after two-day intervals.

The AAFEB reactor failed to sustain dechlorination of PCE. Residual PCE was routinely present in the effluent. Methanogenesis and acetogenesis were the dominant activities within the reactor. The addition of inhibitory levels of ETH (to suppress methanogenesis) did not resurrect the reactor's performance.

## E. CONCLUSIONS

VC concentration alone does not determine ETH production rates. At similar VC concentrations, PCE-fed bottles produced and sustained ETH production while VC-fed bottles

could not. This suggests that the presence of PCE (and /or TCE, DCEs) is (are) required to sustain VC dechlorination.

Results from $H_2$ studies are consistent with a model in which $H_2$ is the electron donor directly used for dechlorination by organisms resistant to vancomycin, and that the role of acetogens in MeOH cultures is to metabolize a portion of the MeOH to $H_2$. MeOH and other substrates shown to support dechlorination in pure and mixed cultures may merely serve as precursors for the formation of an intermediate $H_2$ pool.

The efficiency of our high-PCE/MeOH culture apparently results from the inhibition of MeOH-using methanogens by high PCE (or dechlorination product) concentrations, thus lessening competition for MeOH. When non-inhibitory concentrations (i.e., 9 mg/L) of PCE are employed with the high-PCE/MeOH culture, methanogenic activity rises and consumes an ever-increasing fraction of MeOH equivalents, necessitating ever-greater additions of MeOH to allow sustenance of dechlorination. However, this tactic is a spiral to failure, as ever-greater additions of MeOH support ever-larger methanogenic populations. This same phenomenon may have contributed to the failure of our continuous-flow bioreactors. An additional hypothesis to explain failure of the AAFEB system was loss of dechlorinating biomass from the diatomaceous earth support matrix, due to shear induced by bed expansion.

## F. RECOMMENDATIONS

A potential solution to the problem posed by methanogenic competition would be the use of nonmethanogenic substrates that can act as sources of $H_2$. Ethanol, lactate, and butyrate would be good substrates for investigation; $H_2$ is a direct product of their fermentation to acetic acid (HAc). Since they are not direct methanogenic substrates (ethanol can be used by some methanogens but poorly and not as a direct methane precursor), they may prove to be more efficient suppliers of reducing equivalents for dechlorination, and they are less toxic to humans than MeOH. It remains to be seen with what efficiency our culture might operate on them, and what nutrient deficiencies their use might either cause or eliminate.

Alternative, nonmethanogenic substrates might eliminate direct methanogenic competition for the supplied form of the reductant; however, methanogenic competition for the resulting $H_2$ must still be reckoned with. Possible differences in $H_2$-use thresholds between methanogens and dechlorinators may be exploited to advantage in this context. Possible differences in environmental preferences (e.g., pH) might also be advantageously exploited. Thus, the $H_2$-consumption kinetics of methanogens and dechlorinators should be investigated — including studies of the nutritional and environmental requirements of the organisms. In this manner, strategies may be developed to make optimal use of supplied reductant while minimizing undesirable competing reactions.

In addition to exploring alternative electron donors, investigators should explore alternative reactor support matrices and alternative configurations which may offer superior organism retention in continuous-flow bioreactors.



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# SECTION I

# INTRODUCTION

## A. OBJECTIVE

Tetrachloroethene (also known as perchloroethylene, or PCE) and the less-chlorinated ethenes produced from it via reductive dehalogenation — trichloroethene (TCE), dichloroethene (DCE) isomers and vinyl chloride (VC) — have become common groundwater pollutants throughout the United States, including USAF IRP sites. PCE, unlike less chlorinated ethenes, is not degraded by aerobes; however, reductive dechlorination of PCE by anaerobes has been observed by numerous investigators to varying degrees, sparking interest in developing remediation technologies based upon anaerobic microbial systems.

The promise of anaerobic bioremediation for chlorinated ethenes has not been realized, largely due to a lack of understanding of microbiological fundamentals. Despite nearly a decade of study, we still do not know the roles played by general categories of microorganisms inhabiting mixed-culture, dechlorinating enrichments (methanogens, sulfate reducers, acetogens, etc.). We know little about nutritional and process requirements for dechlorination. Consequently, we cannot explain the observed variability in extent of transformation among many field and laboratory studies.

Specific objectives of this research were:

- To determine if PCE's presence is necessary to sustain dechlorination of VC;
- To delineate the role of hydrogen ($H_2$) in reductive dechlorination of PCE;
- To investigate the ability of our high-level PCE/methanol (MeOH) culture to utilize low levels of PCE — a critical factor in determining success or failure of our proposed bioremediation system;
- To investigate the potential of an Anaerobic Attached-Film Expanded-Bed (AAFEB) reactor to achieve continuous-flow dechlorination of PCE.

## B. BACKGROUND

Under anaerobic conditions, PCE can be completely dechlorinated to ETH by a stepwise, reductive dehalogenation process; however, the final reduction step is apparently rate-limiting, with significant levels of the intermediate VC typically observed. Our first experimental objective derives from our repeated observation that VC dechlorination and ETH production are readily sustained in PCE-fed systems, whereas we have experienced difficulty with VC as the sole

chlorinated specie. PCE's presence may be necessary to induce what is a relatively nonspecific dechlorination activity capable of acting upon many other chlorinated species — including VC.

Dechlorination via a reductive mechanism requires an electron donor. In previous studies conducted under USAF sponsorship (1), we observed that several substrates — glucose, MeOH, acetate (HAc), formate, and $H_2$ — could sustain the reductive dechlorination of PCE. However, the identity of the electron donor directly involved in dechlorination was undetermined. Since several alternate donors appear to serve the dechlorination process, our hypothesis is that the simplest — $H_2$ — may be the donor directly involved. This hypothesis is addressed in our second experimental objective.

In recent, unsponsored studies (2), we developed anaerobic MeOH/PCE enrichment cultures which proved capable of dechlorinating high concentrations of PCE to ETH. Added concentrations of PCE as high as 550 μM (91 mg/liter nominal concentration, or approximately 55 mg/liter actual aqueous concentration) were routinely dechlorinated to 80 percent ETH and 20 percent VC within 2 days at 35°C. This culture dechlorinates PCE at unprecedented, high rates with efficient use of MeOH as the electron donor for reductive dechlorination. The MeOH/PCE culture produced little or no methane ($CH_4$) when high PCE doses were added, presumably because of inhibition of methanogenesis by PCE. What remains to be determined is whether the subsequent use of this high-level culture at lower, noninhibitory concentrations of PCE will spark a rise in methanogenic activity, consuming an ever-increasing fraction of MeOH equivalents, necessitating ever-greater additions of MeOH to allow sustenance of dechlorination. These issues are addressed in our third experimental objective.

Our earlier efforts (3) to achieve continuous-flow dechlorination of PCE met with only limited success; quantitative conversion of PCE to VC resulted, with only minor amounts of ETH produced. We believe that the plug-flow regime associated with our earlier, static-bed reactor does not provide adequate conditions to promote the conversion of VC to ETH. In essence, the auxiliary substrate (in this case, MeOH) is consumed so much more rapidly than is VC, that none of the MeOH reaches the upper zones of the column where it is needed to promote dechlorination of VC. An alternative hypothesis is that PCE (or one of its lesser-chlorinated daughter products, such as TCE or DCE) is needed to induce dechlorinating activity — that VC dechlorination is only a serendipitous occurrence. If so, the plug-flow regime may be inferior because the organisms in the upper zones of the column never "see" PCE or TCE. The AAFEB reactor offers essentially the complete-mix conditions of a suspended-growth system (i.e., a system where we *have* met with significant success), while providing a mean-cell residence time (or "solids retention time", SRT) greatly in excess of the hydraulic retention time (HRT). The AAFEB system was evaluated as part of our fourth experimental objective.

## C. SCOPE

Early experiments concerning the sustenance of dechlorination of VC in the absence of PCE were completed using a methanogenic culture which had demonstrated capability in dechlorinating PCE at low concentrations (0.5 mg/L) to VC and ETH (1). Experiments were completed at 35°C, in either batch or semicontinuous mode with the suspended mixed culture in 160-mL serum bottles. Batch doses of PCE and/or VC were delivered to the cultures and the resultant transformations were monitored using a headspace gas chromatographic technique. In some studies, repetitive additions of PCE and/or VC were administered on a regular basis, over many months — along with MeOH which served as electron donor. The resultant ETH productions of VC-fed systems were compared with those of PCE-fed cultures to determine the need for PCE to sustain complete dechlorination.

Increasing doses of PCE were given to selected cultures to encourage development of a culture in which dechlorination was a major activity. An anaerobic culture (termed "high-PCE/MeOH") with superior PCE reduction capability was developed from this action (2). Selective inhibitors were applied to this culture to elucidate the role of the PCE dechlorinating organism(s) of this culture. $H_2$ and MeOH were employed as electron donors during inhibitor studies to facilitate a more thorough understanding of the roles of major classes of organisms, and to delineate the role of $H_2$ in dechlorination.

The ability of the high-PCE/MeOH culture to sustain dechlorination at low PCE levels was investigated in semicontinuous, serum-bottle studies at 35°C. Two different levels of PCE and three different MeOH:PCE ratios were employed. Over a 34- to 42-day period, the patterns of VC and ETH production following repeated additions of PCE and MeOH were observed and compared.

An AAFEB reactor was designed, constructed and operated over a 200-day period at 35°C. The liquid volume was 1.56 liters; the bed media consisted of 100-μm diatomaceous earth particles. PCE dissolved in a prereduced basal medium was continuously pumped to the reactor from a feed reservoir; a concentrated MeOH/water mixture was continuously injected directly into the bed via a syringe pump. Performance was monitored by headspace gas chromatography and by removal of liquid samples for analysis of nonvolatiles. Several different influent PCE concentrations, MeOH:PCE ratios, and HRTs were explored.

# SECTION II

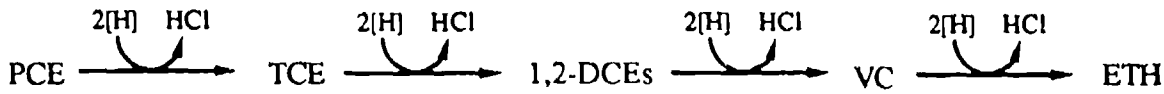
# LITERATURE REVIEW

## A. ANAEROBIC BIOTRANSFORMATION OF PCE

### 1. *In Vivo* Studies

Biotransformation of PCE and TCE under anaerobic conditions has been observed in field studies (4), fixed-film reactors (5, 6, 7, 8, 9, 10), soil (11), sediment (12, 13, 14, 15, 16, 17), aquifer microcosms (18, 19), and to a limited degree in pure cultures (20, 21, 22, 23). A comprehensive review of such studies was prepared for an earlier USAF technical report (3) and will not be repeated here. This present review focuses on results of more recent studies.

Our laboratory was the first to report *complete* dechlorination of PCE to ETH, a process involving sequential reduction steps with TCE, DCE isomers, and VC as intermediates (1):




Though complete, stepwise, reductive dechlorination of PCE to ETH is possible, the final dechlorination step (VC to ETH) appears to be rate-limiting, with significant levels of VC typically persisting (1). Recent reports demonstrate that further reduction to ethane is possible (10, 24, 25).

Dechlorination by a reductive mechanism requires an electron donor. Glucose, HAc, formate, MeOH, and $H_2$ have each been shown capable of sustaining the reductive dechlorination of PCE (1), as have lactate, propionate, crotonate, butyrate, and ethanol (10, 26). More exotic reductants, such as toluene (27) and dichloromethane (28), have also been observed to support dechlorination of PCE. However, the identities of the electron donors *directly* involved in dechlorination were not clear from these previous studies, since large excesses of electron donor were typically provided, with only a minute fraction needed for reductive dechlorination.

For example, our early studies employed 0.5 to 1.0 mg/L PCE concentrations, with 100-fold excesses of electron donor (1). Consequently, the relatively insignificant demand of dechlorination for reducing equivalents could conceivably be satisfied from intermediates and products of the provided electron donor — or even from endogenous sources. Less than 1 percent of the supplied-donor equivalents were used in dechlorination; the remainder appeared as $CH_4$, though the role of methanogens in dechlorination was far from clear or certain. The consequent low fraction of the total microbial activity that could be attributed to dechlorinating activity in these

previous studies also made it impossible to determine the identities of the dechlorinating organisms.

In our early research, MeOH yielded the most complete conversion to ETH; consequently, we focused subsequent studies on this electron donor and succeeded in developing a PCE/MeOH enrichment capable of rapidly dechlorinating high concentrations of PCE to ETH (2). Added PCE concentrations of 550 μM were routinely dechlorinated to 80 percent ETH and 20 percent VC within 2 days at 35°C. The observed transformations occurred in the absence of methanogenesis, which was apparently inhibited by the high concentrations of PCE. An electron balance demonstrated that MeOH consumption was completely accounted for by dechlorination (31 percent) and HAc production (69 percent). The high rates of PCE dechlorination — up to 1.24 mg PCE per mg volatile suspended solids per day (29) — and the relatively large fraction (ca. one-third) of the supplied electron donor used for dechlorination, suggest that reductive dechlorination could be exploited for bioremediation of sites contaminated by chlorinated ethenes.

The promise of anaerobic bioremediation for chlorinated ethenes has not yet been realized, largely due to a lack of understanding of microbiological fundamentals. Despite nearly a decade of study, we still do not know the roles played by general categories of microorganisms inhabiting mixed-culture, dechlorinating enrichments (methanogens, acetogens, etc.). We know little about nutritional and process requirements, including the identities of the immediate reductants in the dechlorination processes. Consequently, we cannot explain the observed variability in extent of transformation among many field and laboratory studies. PCE transformation sometimes stops at TCE (22, 29, 30); in other instances, at DCEs (22, 27) or VC (8, 22); while some field and laboratory studies have demonstrated complete dechlorination to ETH (1, 2, 22, 25). Clearly, process reliability awaits a better understanding of process fundamentals. A better fundamental understanding would also facilitate modeling of the in-situ fate of chlorinated ethenes.

### 2. *In Vitro* Studies

Biochemical studies have been performed by various investigators in an attempt to elucidate the roles of various microorganisms in dechlorination (31, 32). Gantzer and Wackett investigated the ability of several metallocofactors to mediate biological reduction of chlorinated ethenes, and concluded that vitamin $B_{12}$ and coenzyme $F_{430}$ were able to mediate the dechlorination of PCE to ETH, while hematin was capable of dechlorinating PCE only to VC (31). Furthermore, it was observed that the rates of dechlorination mediated by these enzymes significantly decreased as the compounds were transformed into lesser chlorinated ethenes.

The results of these investigations, however, cannot be used to identify the dechlorinating microorganisms within our high-PCE culture, since virtually all of the candidate microorganisms within the mixed culture — methanogens and acetogens, whether MeOH-, HAc-

or $H_2$-users — have many metal porphyrin cofactors in common. Furthermore, the investigations by Gantzer and Wackett infer *cometabolism* as the mechanism involved in dechlorination, and rates of dechlorination measured in their study were similar to those of two pure strains of methanogenic bacteria observed by Fathepure et al. (21). However, in studies involving our high-PCE/MeOH culture, the high specific rate of dechlorination and the zero-order conversion of PCE to VC suggest a metabolic rather than a cometabolic mechanism. Also, the high rate of PCE dechlorination by the high-PCE culture is presumably a result of the exposure of the culture to PCE over a relatively long period of time and an acquired capability of the culture to dechlorinate PCE during its development.

## B. RECENT CONTINUOUS-FLOW, FIXED-FILM STUDIES

Bruin et al. (10) achieved reductive dechlorination of PCE in a continuous-flow, fixed-bed column at 20°C (and later 10°C), inoculated with a mixture of anaerobic sediment from the Rhine River and an anaerobic granular sludge. Lactate (1 mM) was the electron donor; and PCE (8 μM) was completely dechlorinated to ETH and ethane at 10°C, with an HRT of 6 h. Note that the ratio of lactate to PCE employed by Bruin et al. exceeded 50:1 on an electron equivalent basis (assuming lactate $\rightarrow$ HAc + $2H_2$ and PCE $\rightarrow$ ETH). Bruin et al. hypothesized that several different microorganisms are needed to achieve complete dechlorination of PCE. This hypothesis was based upon their development of two different enrichment cultures from the column — one that converted PCE to *cis*-1,2-DCE; and another that converted *cis*-1,2-DCE and VC to ETH.

Chu (33) observed partial dechlorination of PCE in a continuous-flow AAFEB reactor at 35°C. The major dechlorination products were *cis*-1,2-DCE and, presumptively, VC (which was not quantified). HRTs ranged from 1 to 11 hours. Large excesses of sucrose (typically 500 mg/L as COD) were used as electron donor, with influent PCE typically 10 to 20 mg/L. These conditions represent sucrose:PCE equivalent ratios from 130:1 to 65:1, respectively.

Carter and Jewell (34) extended the AAFEB reactor work of Chu to a temperature of 15°C. Influent PCE concentrations were 8 to 12 mg/L; influent sucrose concentration was typically 250 mg/L (as COD) — representing a 65-fold excess of sucrose. Using HRTs of 2 to 4 hours, Carter and Jewell observed ca. 99 percent PCE removal, but 40-50 percent remained as VC in the effluent (with lesser, though significant, concentrations of *cis*-1,2-DCE).

DiStefano explored the ability of a MeOH-fed low-PCE culture to dechlorinate PCE in a continuous-flow, fixed-bed, fixed-film column (3, 29). Influent PCE was 3 mg/L; influent MeOH was 50 mg/L; and an HRT of 2 days was employed at 35°C. PCE was detected in the first 15 percent of the column's length. Thereafter, VC was the only chlorinated species detected. He observed incomplete conversion of PCE to ETH in the column and suggested that the reason for

failure may have been the plug-flow operation of the column; since organisms in the downstream portion of the column, where VC persisted, were never exposed to PCE, DiStefano conjectured that the presence of PCE might be necessary to stimulate dechlorination. Alternatively, the electron donor, MeOH, could have been consumed completely in the upstream section of the column, thereby leaving the downstream organisms with no donor available to convert VC to ETH. Therefore, a completely mixed attached-film reactor system was suggested for future research to expose the entire microbe population to PCE and the electron donor supplied.

# SECTION III

# MATERIALS AND METHODS

This section describes materials and methods common to many of the individual studies which constituted this research project. Procedures unique to particular studies are described in subsequent sections of this report.

## A. PROGRAM OF STUDY

Initial experiments focused on the final transformation step of dechlorination, VC conversion to ETH. The "low-level" PCE/MeOH culture originally developed by Freedman was employed (1, 28) at nominal PCE concentrations of 0.5-0.6 mg/L. MeOH served as the electron donor, and various serum bottles were prepared to observe production of ETH in the presence of PCE and VC, and VC alone. Such experimentation was undertaken to determine if PCE's presence was required to achieve dechlorination of VC to ETH.

Subsequent experiments were conducted with the "high-level" PCE/MeOH culture developed by DiStefano (2, 29) using nominal PCE concentrations of 550 μM (91 mg/L). A kinetics experiment was completed to determine maximum specific rates of PCE transformation and to observe the production and decline of intermediates. $H_2$ was applied as an alternate electron donor to facilitate an understanding of its possible role as the direct electron donor for PCE reduction. Selective inhibitors (vancomycin and bromoethanesulfonic acid) were employed to examine the roles of the major classes of organisms in $H_2$- and MeOH-fed systems degrading high PCE concentrations.

Since many contaminated groundwaters contain concentrations of PCE which are significantly lower than 550 μM, it was desired to undertake studies which explored the capability of this high PCE culture to sustain dechlorination of lower levels of PCE. Two goals were identified: (1) To determine the ability of the high-PCE enrichment culture to sustain dechlorination of low concentrations of PCE through a long-term bottle study, where MeOH was employed as an electron donor at varying MeOH:PCE ratios; and (2) To design, construct, and maintain a continuous-flow AAFEB reactor and observe the ability of the high-PCE enrichment culture to dechlorinate PCE at low concentrations, if supplied with MeOH at an optimal MeOH:PCE ratio. The basic experimental approach involved inoculation of serum bottles and a column reactor with the high-PCE enrichment culture, followed by a feeding of appropriate amounts of PCE, electron donor, and nutrient source to each culture on a regular basis. PCE transformation and $CH_4$ formation were measured from headspace samples which were analyzed

with a gas chromatographic technique, and either a liquid chromatographic method or an enzymatic method involving spectrophotometry was employed to measure non-volatile, soluble compounds. Bottles were maintained semicontinuously, and the column reactor was operated in batch, semicontinuous, and continuous modes throughout the course of the experiments. All experiments were conducted at 35°C.

## B. CHEMICALS AND STOCK SOLUTIONS

PCE, TCE, *trans*-1,2-DCE, *cis*-1,2-DCE, and 1,1-DCE were obtained in neat liquid form (1- or 5-gram ampoules; Supelco, Inc.) for use as analytical standards. High-performance-liquid-chromatography (HPLC)-grade PCE (99.9+ percent; Aldrich Chemical Co.) and HPLC-grade MeOH (99.9 percent; Fisher Scientific) were used as culture substrates. $H_2$ (99.95 percent minimum purity) also served as a culture substrate and was purchased from Linde Specialty Gases and Equipment. VC, ETH, and $CH_4$ were obtained as gases in lecture bottles (99+ percent; Scott Specialty Gases). $H_2$ (1 percent in $N_2$) was purchased from Airco, Inc. for use as a low-concentration standard with the Reduction Gas Detector. Dimethyl sulfide (DMS, 99+ percent) was obtained from Aldrich Chemical Co. Vancomycin hydrochloride (963 µg/mg) and 2-mercaptoethanesulfonic acid (Coenzyme M or 'CoM') were purchased from Sigma Chemical Company. 2-Bromoethanesulfonic acid (BES) was obtained from Aldrich Chemical Co.

Experiments often employed a 25-percent aqueous stock solution of MeOH and a yeast extract (YE, 50 g/L) stock solution. The stocks were added to serum bottles and gassed with 70 percent $N_2$-30 percent $CO_2$ via cannula prior to application of a butyl-rubber stopper and aluminum crimp cap. For experiments in which a low level of electron donor was required, the stock solution of YE was inoculated with 10 mL of the high-PCE culture and incubated for ten days at 35°C in order to ferment much of the organic content of the YE. This fermentation process created a stock solution containing necessary nutrients but relatively little organic matter which might provide electron equivalents when added to cultures. Low levels of PCE were delivered from a distilled-water stock which was saturated with PCE. In studies with high levels of PCE, neat PCE was added by syringe to the cultures; the stock of neat PCE was not purged with $N_2$-$CO_2$.

Alcohol oxidase (from *Pichia pastoris*, phosphate-buffered 60 percent sucrose solution), peroxidase (Type I from horseradish) and 2,2'-azino-di-(3-ethyl)-benzthiazoline-6-sulfonic acid (ABTS, 98 percent, diammonium salt) were used in the preparation of an enzymatic reagent for the analysis of MeOH (all chemicals from Sigma Chemical Co.).

A reducing solution containing titanous chloride (20 percent, Fisher Scientific), sodium bicarbonate (Fisher Scientific) and citric acid, trisodium salt dihydrate (99 percent, Aldrich

Chemical Co.) was used as a scrubbing agent to remove trace levels of oxygen in the 30 percent $CO_2$/70 percent $N_2$ purge-gas mixture described for use in various procedures of this research.

## C. CULTURES AND ENRICHMENT PROCEDURES

### 1. General Procedures

All experiments were conducted using 100-mL culture volumes in 160-mL serum bottles. As discussed later in this chapter, two 6-liter stock cultures were maintained to serve as sources of organisms. In preparation for experiments, 100-mL mixed-liquor aliquots were anaerobically transferred directly from the reactor of choice to serum bottles via a Unispense II pump. At least 2 minutes before dispensing culture, serum bottles were made anaerobic by adding a cannula flowing with 70 percent $N_2$-30 percent $CO_2$. Upstream from the cannula, the $N_2$-$CO_2$ mixture was continuously bubbled through a titanium-chloride solution to remove trace levels of oxygen. Maintenance of serum-bottle cultures was dependent on objectives of individual experiments. Therefore, details concerning culture maintenance accompany descriptions of respective experiments.

Experiments involving low-level (methanogenic) PCE cultures utilized Teflon®-lined natural rubber septa (Supelco) held in place by aluminum crimp caps. Later, Teflon®-lined, butyl-rubber septa (Wheaton) were judged superior in withstanding multiple needle punctures. Therefore, the butyl-rubber septa were used for subsequent experiments involving high-level (non-methanogenic) anaerobic cultures. In all cases, the bottles were maintained at 35°C in an inverted position to minimize loss of volatile compounds. Unless otherwise noted, bottles which received MeOH were incubated quiescently, whereas $H_2$-fed bottles were incubated in an orbital shaker bath to facilitate transfer of $H_2$ to the liquid phase. Cultures were grown in a basal salts medium with a modified (1, 28) composition from that of Zeikus (35). The basal medium recipe is shown in Table 1.

### 2. Low-Level (Methanogenic) PCE Stock Culture

Methanogenic mixed cultures were obtained from those developed by David Freedman (1, 28). Freedman's culture was added to basal medium in a 9.6-liter bottle (Fisher Scientific) to develop a large-quantity source of organisms for future experiments. A 6-liter liquid volume was chosen to result in an identical liquid volume/total volume ratio as in the 160-mL serum bottles. Headspace conversion factors from the 160-mL serum bottles were thus applicable to the 9.6-liter bottle during routine analysis of volatile components. Figure 1 is a schematic of the 6-liter reactor.

TABLE 1. BASAL MEDIUM FOR ENRICHMENT CULTURES

| Compound* | Quantity |
|---|---|
| $NH_4Cl$ | 0.20 g |
| $K_2HPO_4 \cdot 3H_2O$ | 0.10 g |
| $KH_2PO_4$ | 0.055 g |
| $MgCl_2 \cdot 6H_2O$ | 0.20 g |
| Resazurin | 1 mg |
| Trace Metals Solution** | 10 mL |
| $FeCl_2 \cdot 4H_2O$ | 0.10 g |
| $Na_2S \cdot 9H_2O$ | 0.50 g |
| $NaHCO_3$ | 6.0 g |
| Distilled-deionized water | 1000 mL |

* First seven items were added, $N_2$ purge was maintained until solution changed from blue to pink, remaining items were added, and then purge gas was switched to 30% $CO_2$/70% $N_2$. HCl was added as needed to achieve pH 7.

**0.1 g/L $MnCl_2 \cdot 4H_2O$; 0.17 g/L $CoCl_2 \cdot 6H_2O$; 0.10 g/L $ZnCl_2$; 0.20 g/L $CaCl_2$; 0.019 g/L $H_3BO_4$; 0.05 g/L $NiCl_2 \cdot 6H_2O$; 0.02 g/L $Na_2MoO_4 \cdot 2H_2O$. Adjust to pH 7 with NaOH or HCl.

PCE and VC retention in the 9.6-liter bottle was examined prior to addition of culture and basal medium. 21 µmol PCE and 18 µmol VC were added to 6 liters of distilled water, and the bottle was sealed and maintained at 35°C on its side. After 4 days, essentially all the added PCE was measured and 85 percent of the added VC remained. The design was deemed acceptable, since the biological transformation of PCE to VC occurred at a much faster rate than the observed VC loss rate; furthermore, the 9.6-liter bottle was *only* intended as a reservoir of organisms. All experiments would be conducted with organisms in 160-mL serum bottles.

Every second day, the culture was fed (via syringe), 16 mmol MeOH (87 mg/L), 18 µmol PCE (0.5 mg/L nominal liquid concentration, ignoring partitioning to the headspace) and 12 mg YE (2 mg/L). A PCE/MeOH stock was prepared by dissolving neat PCE in MeOH. The YE stock in distilled water was prepared in a serum bottle and purged thoroughly with 70 percent $N_2$-30 percent $CO_2$ prior to crimp-sealing with a butyl-rubber septum. Because of the relatively low PCE dose, this methanogenic master culture was termed the "low-PCE culture." Before feeding, headspace analysis was completed, and gas production was measured and vented. Gas production was measured by connecting tubing between the reactor and a calibrated gas

displacement bottle filled with distilled water. Following gas volume measurement, 800 mL of basal medium was added, the bottle was vigorously mixed, and 800 mL was then wasted from the 9.6-liter bottle. Basal medium was added by connecting, with Tygon® tubing, the reactor to a reservoir of basal medium; the reservoir was pressurized with 70 percent $N_2$-30 percent $CO_2$, and basal medium was transferred to the reactor. The reactor was shaken vigorously, and then mixed liquor was wasted through the stainless-steel valve to a graduated cylinder. The feeding and wasting procedure occurred every second day, thus resulting in a nominal HRT of 15 days.

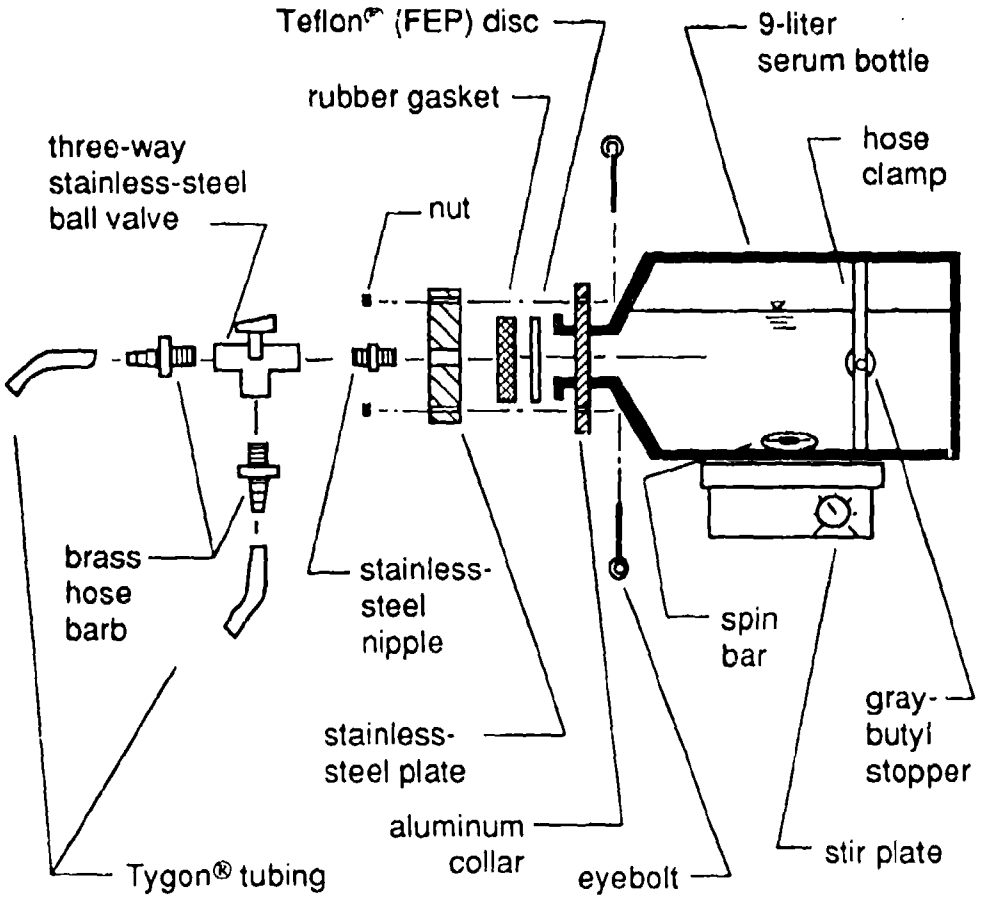



Figure 1. Schematic of 6-Liter Reactor (Exploded View).

### 3. High-Level (Nonmethanogenic) PCE Stock Culture

Two 160-mL serum bottles containing methanogenic (low-PCE) culture were fed ever-increasing PCE doses to investigate tolerance to PCE. When the PCE dose reached about 25 µmol/100 mL (33 mg/L), $CH_4$ production declined and eventually ceased; improved conversion of PCE to ETH was also observed. Eventually, the nominal PCE dose reached 55 µmol/100 mL (91 mg/L) and was routinely restored to 55 µmol following consumption. Details concerning the transformation of this culture are given elsewhere (2). This transformation was considered significant; we therefore proceeded to prepare a large-volume source of this culture for subsequent experiments. A second 9.6-liter bottle, identical to that depicted in Figure 1, was constructed and seeded from the two serum bottles which had routinely received 55 µmol PCE/100 mL. Basal medium (5.8 liters, Table 1) was prepared in the 9.6-liter bottle and culture from the two serum bottles was transferred via gas-tight syringe to the large bottle. The resultant liquid volume of the reactor was 6 liters.

This anaerobic (essentially nonmethanogenic) 6-liter master culture became known as the "high-PCE" culture. It was maintained as follows: every second day, a headspace sample taken from the reactor was analyzed using gas chromatography in order to measure the production of volatiles, and gas production was vented. The culture was then fed individually, via syringe, the following: 9.4 mmol neat MeOH (50 mg/L), 3.3 mmol neat PCE (91 mg/L), and 120 mg YE (20 mg/L). On every fourth day, after gas production of volatile compounds was measured and vented, the reactor was purged of volatile products with a 70 percent $N_2$-30 percent $CO_2$ gaseous mixture which was passed through a titanous chloride scrubbing solution in order to remove trace levels of oxygen. The anoxic purge gas was delivered to the reactor via two cannula which were inserted into a port located on the side of the reactor. After the purging of the reactor, 600 mL of fresh basal salts medium was anoxically added to the bottle; then the entire reactor was shaken vigorously and was wasted of 600 mL of culture medium, resulting in a nominal HRT of 40 days. The mixed liquor was wasted from the reactor through a three-way stainless-steel valve into a 1000-liter graduated cylinder. Basal medium was transferred to the reactor from a 15-liter reservoir via a connection of Tygon® tubing leading from the reservoir to the reactor. A $N_2/CO_2$ anoxic gaseous mixture delivered to the basal medium reservoir provided the driving force necessary to transfer the medium into the 6-liter reactor. Feeding of the reactor was then completed as on the second day of the 4-day cycle.

## D. ANALYTICAL METHODS

### 1. Gas Chromatography (GC)

#### a. Headspace Analysis of Volatile Organics and Hydrogen.

The total mass of each volatile organic (PCE, TCE, DCE isomers, VC, ETH, $CH_4$, and DMS) and $H_2$ within a serum-bottle culture was determined by a method involving a single 0.5-mL headspace gas injection into a complex network involving simultaneous use of two gas chromatographs (Perkin Elmer-Model 8500), two analytical columns, two multi-port air-actuated switching valves (Valco, Inc.), two flame-ionization detectors (FIDs), and one reduction-gas detector (Trace Analytical RGD). Figure 2 is a schematic of the GC system.

One GC, where injection was made, was equipped with one FID and two columns — a 3.2-mm x 2.44-meter stainless-steel column packed with 1 percent SP-1000 on 60/80 Carbopack®-B, in series with a 3.2-mm x 3.2-meter stainless-steel column packed with 100/120 Carbosieve® G (Supelco, Inc.), with the two switching valves situated upstream and downstream of the Carbosieve® column. For 1.5 minutes after sample injection, flow was routed from the injector through the Carbopack® column to the Carbosieve® column. Within that 1.5-minute period, $H_2$, $CH_4$, and ETH passed through the Carbopack® column, relatively unretained, and into the Carbosieve® column, where they could be resolved. At 1.5 minutes, the Carbopack® column effluent was redirected via switching valve 1 to the FID for eventual detection of VC, DCEs, TCE, PCE, and DMS. For the first 2 minutes following injection, effluent from the Carbosieve® column was routed via switching valve 2 to the RGD for eventual $H_2$ detection. At 2 minutes — just after $H_2$ was detected, but before $CH_4$ eluted — the effluent from the Carbosieve® column was redirected to the FID of the second gas chromatograph for eventual detection of $CH_4$ and ETH. Output from each FID was sent to separate calculating integrators and printers (Perkin Elmer GP-100). Output from the RGD was sent to a calculating integrator (Perkin Elmer LCI 100). $N_2$ served as the carrier gas (30 mL/min through each branch of the network at all times). The columns were in a single oven, which was temperature-programmed as follows: 100°C for 4 minutes; ramped at 20°C/min to 200°C; and held for 8 minutes.

This GC method was used in its entirety when detection of low levels of $H_2$ was desired (i.e., less than about 1,000 nmoles per serum bottle). When RGD measurement of $H_2$ was either not possible (i.e., at $H_2$ > 1,000 nmol/bottle) — or not desired — diversion to the RGD was omitted. In such cases, Carbosieve® flow was constantly routed via valve 2 to FID 2 for detection of $CH_4$ and ETH.

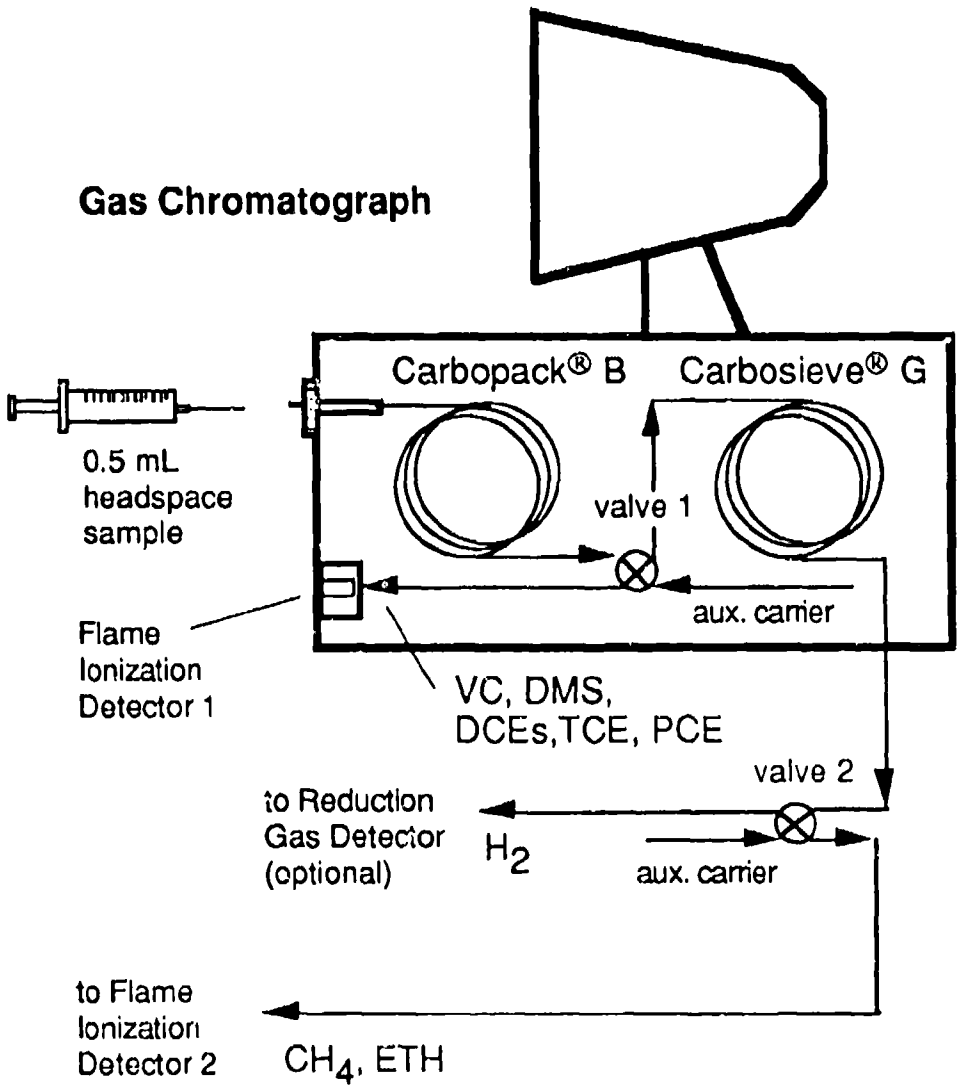



Figure 2. Schematic of Gas Chromatographic System for Headspace Analysis of Volatile Organics ($H_2$ Analysis via Reduction Gas Detector Was Optional).

Initial identification and confirmation of volatile organics was presented previously (1). Subsequent identification was confirmed by injection of standards into the GC system described. GC calibration factors, resulting from a 0.5-mL headspace injection, were related to the total mass of compound present in a serum bottle. Each neat compound was transferred to a known quantity of MeOH in a sealed, 14-mL vial. Quantities of compound and

MeOH were determined gravimetrically. A known mass of compound (in MeOH) was determined gravimetrically and delivered by syringe to a 160-mL serum bottle. The serum bottle contained 100 mL distilled, deionized (DDI) water and was sealed with a Teflon®-lined natural rubber septum or Teflon®-lined butyl-rubber septum and aluminum crimp cap. The bottle was equilibrated at 35°C then analyzed via 0.5-mL headspace injection to the GC.

Since VC, ETH, $H_2$, and $CH_4$ were obtained as gases, volumetric additions were made directly to the 160-mL serum bottles filled with 100 mL DDI water. Standard mass additions to the serum bottles were determined from the ideal gas law. The standards prepared for the calibration of low levels of $H_2$ for analysis by the RGD needed to be purged of even trace levels of $H_2$ before low-level standards (less than 3 nanomoles per 0.5-mL injection) were prepared. Therefore, these standards were purged with prepurified nitrogen (Linde Specialty Gases and Equipment) which was sent through a catalytic combustion filter (Trace Analytical) and then through a molecular sieve (Supelco, Inc.) before entering the calibration-standard bottles through small glass dispersion spargers (Ace Glass, Inc.). $H_2$ (1 percent in a $N_2$ gas mixture) was then added via gas-tight syringes to the bottles.

Higher levels of $H_2$ (i.e., > 1,000 nmol/bottle) were routinely measured by a GC technique involving injection of a 0.5-mL headspace sample into a 3.2-mm x 3.05-meter stainless-steel column packed with 100/120 Carbosieve® S II coupled with a thermal conductivity detector. $N_2$ served as the carrier gas. The GC oven was held at 150°C for 1.5 minutes for this analysis. Standards were prepared as described previously.

The coefficient of variation was used as the measure of precision for standards analysis. Coefficients of variation (standard deviation/mean*100) for calibration factors (relating total mass of each volatile in a bottle to GC peak-area response) ranged from 0.59 percent to 2.8 percent.

**b. GC Analysis of Methanol and Acetate.**

MeOH and HAc analyses were completed by GC using a Perkin-Elmer 8500 GC. A 0.5-µL aqueous injection was made to a 0.53-mm x 15-meter Nukol® fused-silica capillary column (Supelco, Inc.) connected to the FID. The oven temperature was programmed as follows: 100°C for 3 minutes; ramp at 20°C/min to 160°C.

Samples (100-200 µL) were filtered through a 0.45-µm syringe filter (Gelman Sciences) and acidified with 5 µL 2N HCl prior to injection. Samples were sometimes stored in the refrigerator for up to 4 days prior to analysis. To examine the suitability of this storage method, standards were prepared, acidified, analyzed, placed in the refrigerator for 2 weeks, and analyzed again. There was no significant difference between pre- and post-storage analysis. Standard calibration curves were developed for MeOH and HAc. Peak-area responses

from subsequent samples were compared to the standard curves to obtain MeOH and HAc concentrations.

### 2. Acetate and Formate via High Performance Liquid Chromatography (HPLC)

HAc and formate analyses were performed using a Hewlett Packard 1090 HPLC, coupled with a 300-mm HPX-87H ion-exchange column (Bio-Rad Laboratories). When only HAc was to be quantified, a 100-mm x 7.8-mm Fast-Acid (Bio-Rad Laboratories) column was substituted. The Fast-Acid column, though incapable of resolving formate and HAc, was preferred due its shorter run time when HAc was the only organic acid to be measured. Flow from the column in use was directed to a Refractive Index Detector (Perkin-Elmer LC-25) whose response was processed by a calculating integrator (Perkin-Elmer LCI-100). 100-µL samples were used for all HPLC analyses. $H_2SO_4$ (0.013 N) served as the mobile phase and was pumped through the 300-mm column at 0.7 mL/min and through the Fast-Acid column at 0.8 mL/min. The 300-mm column was fitted with a water jacket which held the temperature at 65°C while the Fast-Acid column was used at ambient temperature.

Sample preparation included filtration of a 0.5-mL aliquot (using a Gelman Science's 0.45-µm filter disk), acidification (with 10 µL of 2N HCl), and refrigeration. Standard calibration curves were developed for formate and HAc. Peak-area responses from subsequent samples were compared to the standard curves to obtain formate and HAc concentrations.

# SECTION IV

# TRANSFORMATION OF VC TO ETH IN METHANOGENIC CULTURES

## A. CONTEXT

Figure 3 presents results from a typical "low-PCE/MeOH" enrichment culture (29) — a culture which received noninhibitory levels of PCE, large excesses of MeOH, and in which methanogenesis was therefore the dominant fate of equivalents provided from MeOH. It depicts performance of a 160-mL serum-bottle culture (100-mL liquid volume) operated semicontinuously as follows: Every fourth day, 4 mL mixed liquor was drawn (via syringe) and wasted, and 3.7 mL basal medium was added, plus 0.3 mL (0.3-0.35 µmol or 0.5-0.6 mg/L nominal dose) distilled-deionized water saturated with PCE. The HRT in these bottles was therefore maintained at 100 days. After wasting, basal medium addition, and PCE addition, 156 µmol (50 mg/L) MeOH and 5 mg (50 mg/L) YE were added.

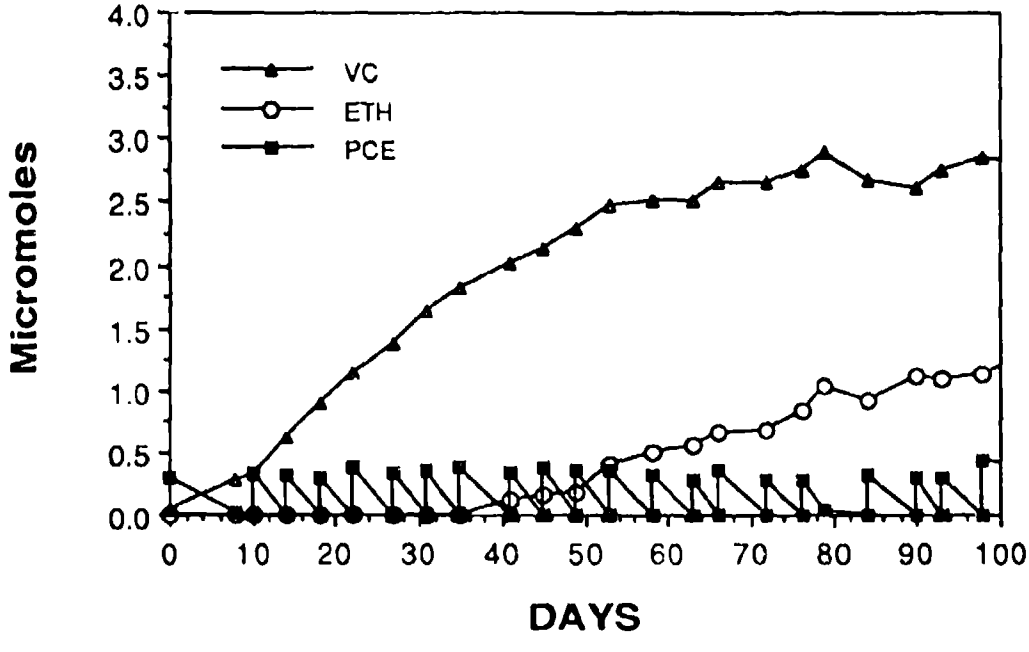



Figure 3. PCE Transformation to VC and ETH in a MeOH-Enrichment Culture.

The performance depicted in Figure 3 is typical in that PCE was completely transformed to VC and ETH during each 4-day incubation period. Each PCE dose was transformed to primarily VC and lesser amounts of ETH. This pattern continued, in that VC persisted and was not completely transformed to ETH. TCE and DCE isomers were rarely detected. Routine $CH_4$ production per feeding ranged from 100 to 120 μmol. Actual $CH_4$ yield was therefore essentially equal to theoretical yield according to the reaction:

$$CH_3OH \rightarrow \frac{1}{4}CO_2 + \frac{3}{4}CH_4 + \frac{1}{2}H_2O.$$

Little MeOH was used in PCE reduction since MeOH was provided in amounts (936 μeq) far in excess of that needed for complete PCE transformation to ETH (2.8 μeq). This master culture was clearly dominated by methanogenic organisms.

Results shown in Figure 3 and presented by Freedman (1, 28) make it clear that considerable levels of VC persist during PCE dechlorination. As discussed in Section II, VC also persisted in a fixed-film plug-flow reactor, possibly because organisms in the mid- and end-sections of the reactor were never exposed to PCE. Therefore, an experiment was performed to determine if PCE was needed to sustain VC conversion to ETH.

## B. PROCEDURES

Six 160-mL serum bottles were prepared from the methanogenic "low-PCE/MeOH" 6-liter master culture; 100 mL mixed-liquor culture was anaerobically transferred to each serum bottle using a Unispense-II pump. Initially, bottles were operated identically to ensure uniform PCE-dechlorinating capability. For 25 days, all bottles were performing similarly; nearly identical amounts of VC and ETH had been produced per PCE addition. At 25 days, four bottles were switched to VC and two bottles continued to receive PCE. Throughout the experiment, a large, common background level of VC (3 to 5 μmol) was maintained in all bottles. Therefore, potential differences in VC transformation to ETH could not be explained by differences in VC among bottles.

Headspace analyses and wasting/feeding operations were completed every 2nd day. Two milliliters of mixed liquor was wasted, 1.7 mL of basal-salts medium (spiked with MeOH and YE) was added, and then followed by 0.3 mL distilled-deionized water saturated with PCE. This represented a PCE dose of 0.35 μmol (0.6 mg/L nominal concentration). MeOH and YE were added to the stock basal medium so that a 1.7-mL aliquot would provide doses of 31 μmol (10 mg/L) MeOH and 0.2 mg (2 mg/L) YE. The nominal retention time in the bottles was 100 days.

From Days 0 to 25, all bottles were maintained in this manner, and each performed similarly. By Day 25, cumulative PCE added was about 4.5 µmol, cumulative VC was approximately 3 µmol and the balance was ETH. Also, near-stoichiometric conversion of MeOH to $CH_4$ was observed during each incubation interval; 20 to 25 µmol $CH_4$ was produced per feeding event.

Throughout this entire experiment (Day 0 to Day 160), regardless of the treatment, all bottles exhibited near-stoichiometric conversion of MeOH to $CH_4$. $CH_4$ production is not presented graphically. Although the MeOH dose was reduced 5-fold compared to previous operations, it was still provided (187 µeq) in excess of that needed for complete PCE reduction to ETH (2.8 µeq).

From Day 25, four bottles were designated to receive routine VC additions instead of PCE during the feeding operation. The remaining two bottles were to continue receiving PCE. The retention time (100 days), MeOH (10 mg/L), and YE (2 mg/L) doses were identical in all bottles. On Day 25, all bottles were purged with 70 percent $N_2$-30 percent $CO_2$, the septa were changed, and about 3.1 µmol VC (gaseous) was added to each bottle. VC was taken from a lecture bottle and delivered to the serum bottles via gas-tight syringe. Two of the six bottles were designated to continue receiving routine PCE doses of 0.35 µmol. The VC-only bottles received about 0.35 µmol VC instead of PCE. VC was added in gaseous form; to maintain the 100-day HRT in the VC-fed bottles, the wasted mixed liquor volume (2 mL) was replaced with 2 mL basal medium.

Routine VC additions were delivered as 0.2-mL pulses from a locking gas-tight syringe (Dynatech Precision Instruments). To facilitate delivery of such a small volume, a stock dilution was prepared by adding 14 mL VC from a lecture bottle to a 160-mL serum bottle containing 100 mL basal medium; the headspace was composed of 70 percent $N_2$-30 percent $CO_2$. Use of basal medium was desirable since it contained resazurin; an increase in reduction/oxidation potential would eventually be detected by a color change from clear to pink, thereby imparting some degree of safety against injection of oxygen to the experimental bottles. This stock VC bottle was kept at 35°C which resulted in a VC headspace concentration of 0.35 µmol per 0.2 mL. The stock VC bottle was also occasionally used to maintain similar background levels of VC in all bottles.

After PCE addition, the bottles were placed inverted in a 35°C water bath and equilibrated for 20 minutes (PCE transformation to TCE was observed when 20 minutes was exceeded). The headspace was then sampled for verification of the quantity of PCE added. Since VC was not transformed as rapidly as PCE, VC-spiked bottles were shaken vigorously and placed in the water bath for 60 minutes before headspace sampling.

## C. RESULTS

Throughout the experiment, replicate bottles performed similarly, therefore, an exemplary bottle from each replicate pair is presented. Figure 4 shows performances of PCE-fed and VC-fed bottles. The Figure includes PCE, VC, and ETH levels; TCE was never observed during routine sampling and DCEs were observed on occasion at insignificant levels. Cumulative PCE added is also included.

Since it was not detected after 2 days' incubation, 0.35 µmol PCE was added to the PCE-fed bottles every 2nd day. These repetitive additions are represented by the "sawtooth" line on the upper graph of Figure 4. Septa were repeatedly punctured for about 30 days before changing, at which point the bottles were purged, and VC was restored. Such purging and septum-changing events are noted in the Figure. The lower graph includes measured VC and ETH levels and cumulative VC added. Initially, 0.35 µmol VC was added as necessary to maintain VC levels at 3 µmol. However, as the experiment progressed, VC in the PCE-fed bottles began to decline. In order to maintain similar levels in all bottles, VC additions to the VC-fed bottles were then stopped, as represented by the horizontal "cumulative VC fed" line from Days 61 to 83.

In PCE-fed bottles, ETH production was fairly constant throughout each operating-purging cycle, and this level of operation appeared sustainable. In contrast, VC-fed bottles (lower graph of Figure 4) exhibited a declining ETH production over successive operating-purging cycles. Figure 5 provides a comparison of ETH production rates in the PCE-fed and VC-fed bottles. VC levels are included for both bottle-types to demonstrate that VC was similar both. Figure 5 shows that the PCE-fed bottles produced about 0.1 to 0.12 µmol ETH per day. Further, this rate of ETH production was fairly constant and apparently sustainable. In comparison, the Figure shows the decreasing ETH production rate in the VC-fed bottles. The rate decreased from 0.1 to about 0.02 µmol ETH per day over the 75-day sampling interval. Apparently, the presence of PCE and/or its lesser chlorinated product(s) (TCE, DCEs) is (are) needed to sustain transformation of VC to ETH.

To examine this further, the four VC-fed bottles were paired so that two individual operating procedures could be compared. One pair was designated to resume routine PCE additions while the remaining pair would continue to receive only routine VC additions. The intent was to see if VC transformation to ETH could be restored by PCE.

Figures 6 and 7 are presented in similar format to that of Figure 4. Figure 6 depicts results from a bottle in which routine PCE additions were resumed from Day 67 to Day 123. An initial lag in PCE transformation is evident in the "sawtooth" line from Days 67 to 83. Thereafter, the routine PCE spikes were completely transformed in 2 days. A decline in ETH production is evident in the second operating/purging cycle. However, an increase in ETH production is observed from Days 85 to 125. PCE additions stopped after Day 125 and a decline in ETH production is noticed.

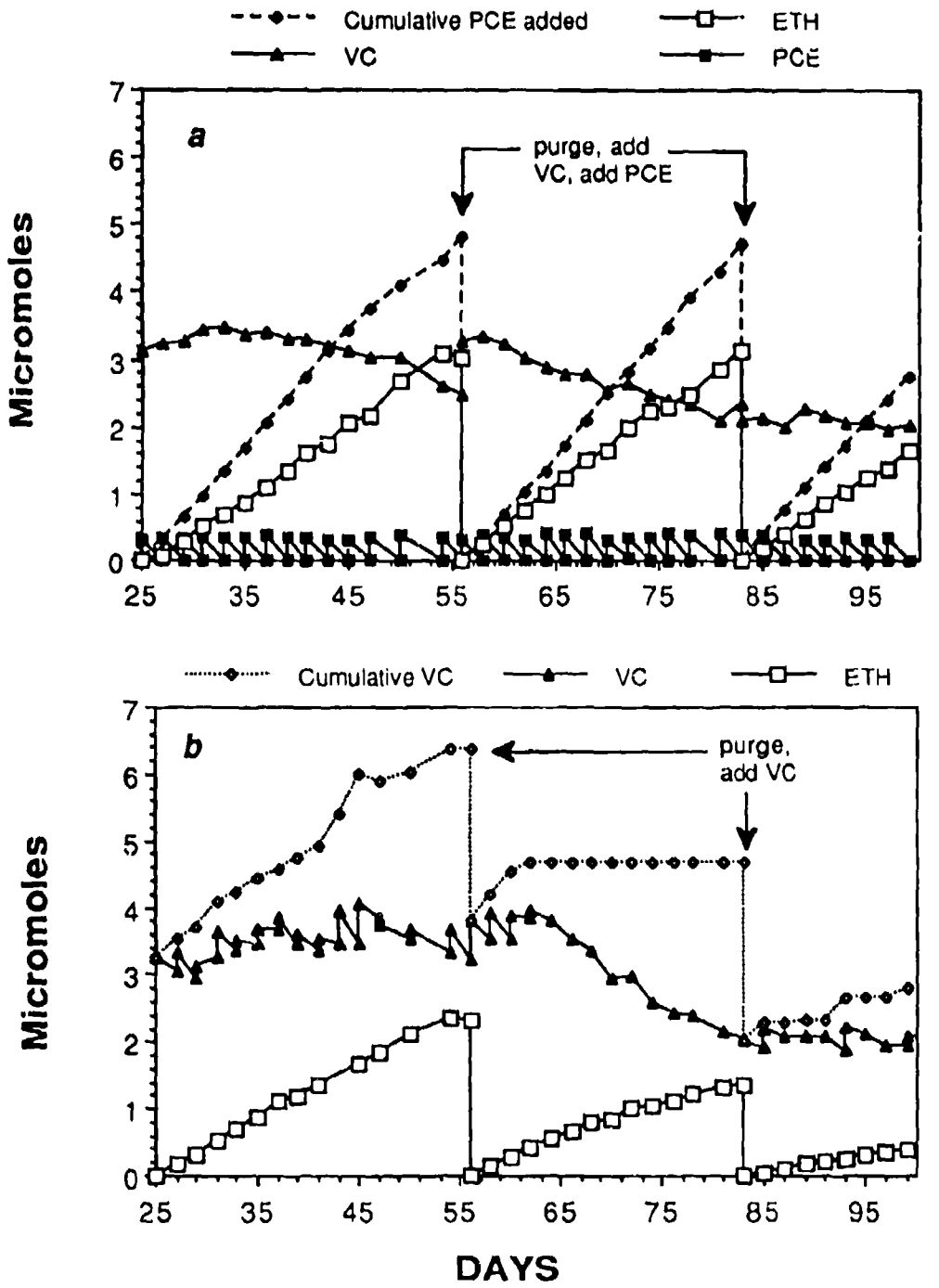



Figure 4. ETH Production in Cultures Fed (a) PCE or (b) VC.

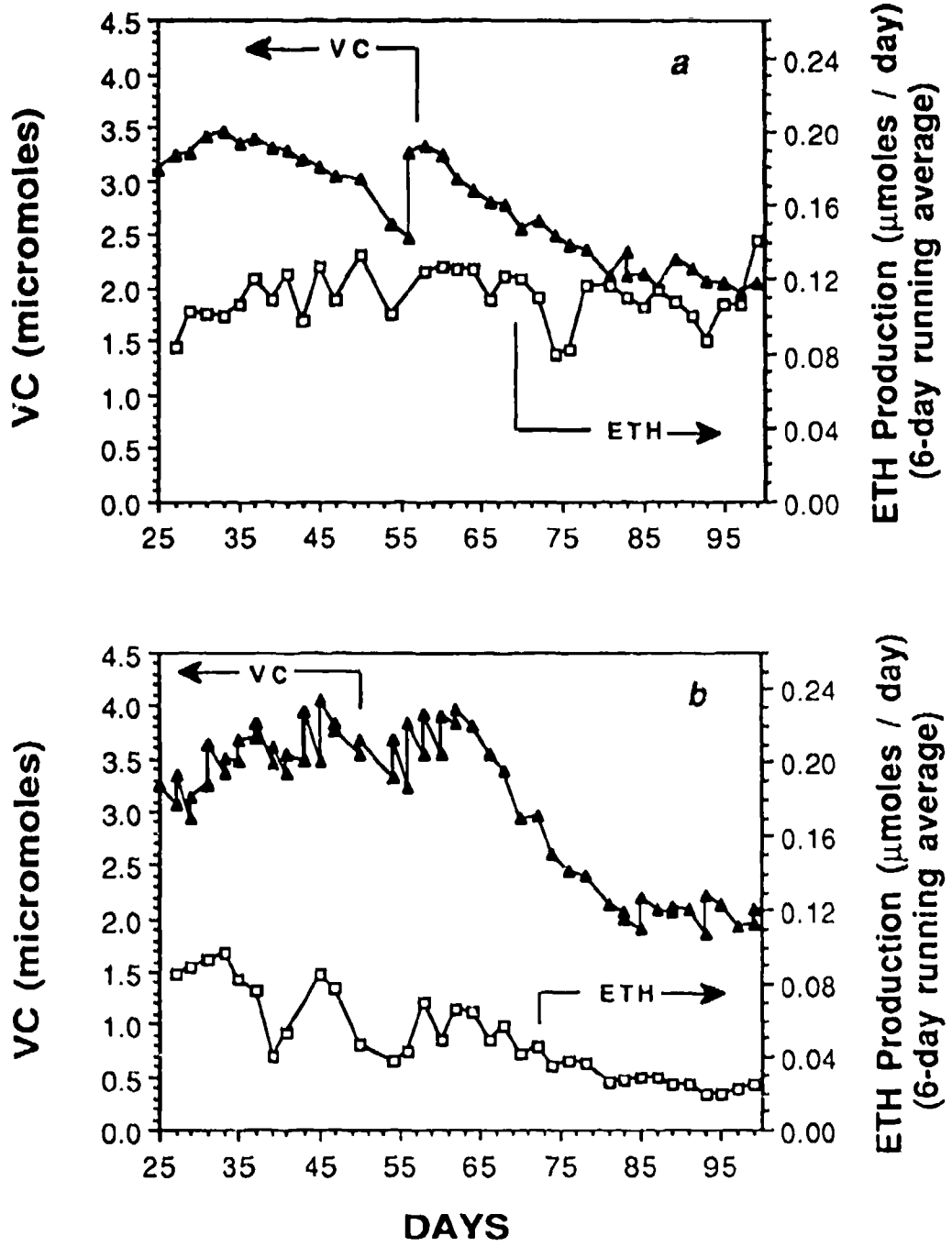



Figure 5. ETH Production Rates in (a) PCE-Fed and (b) VC-Fed Bottles.

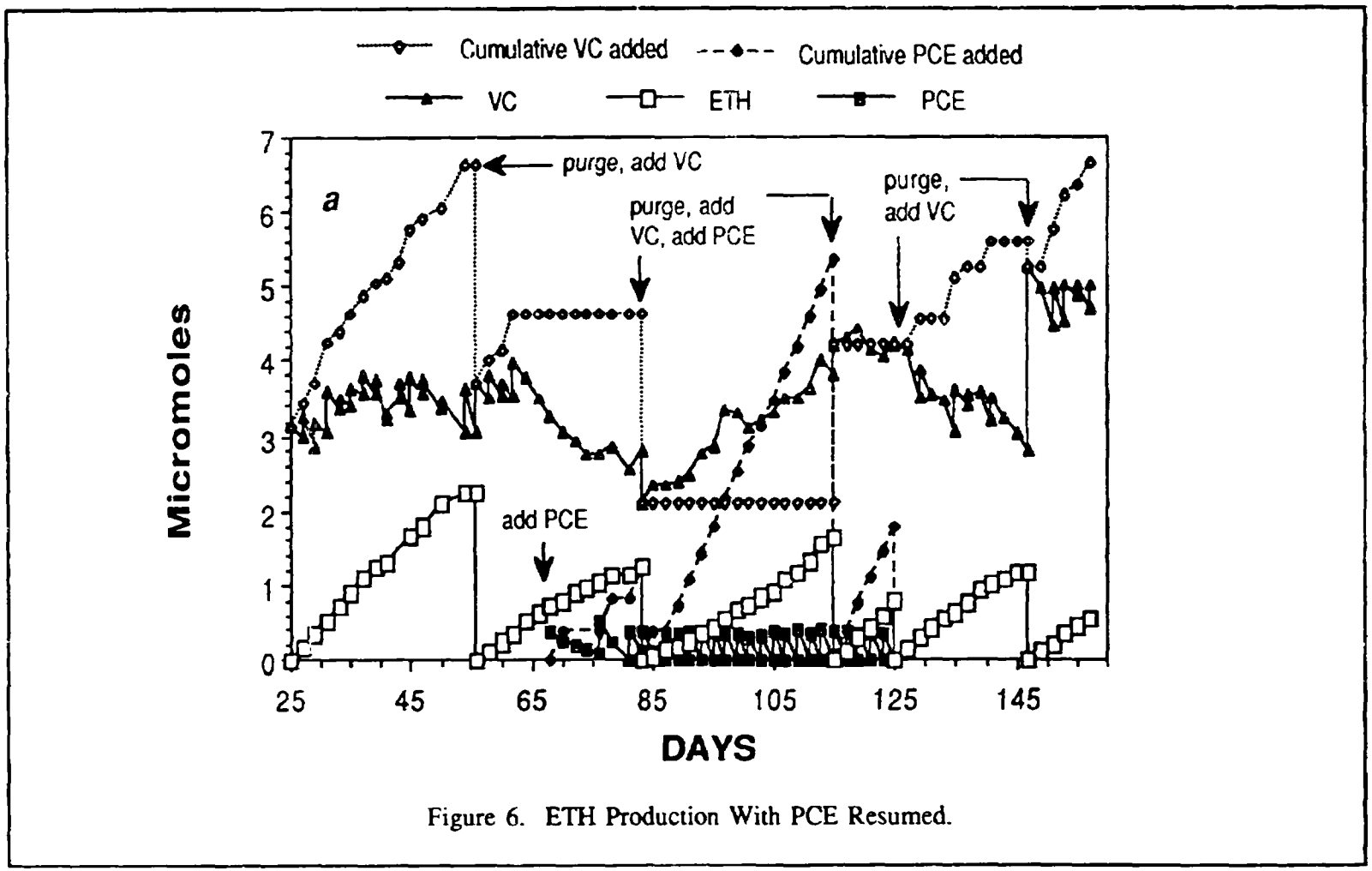



Figure 6. ETH Production With PCE Resumed.

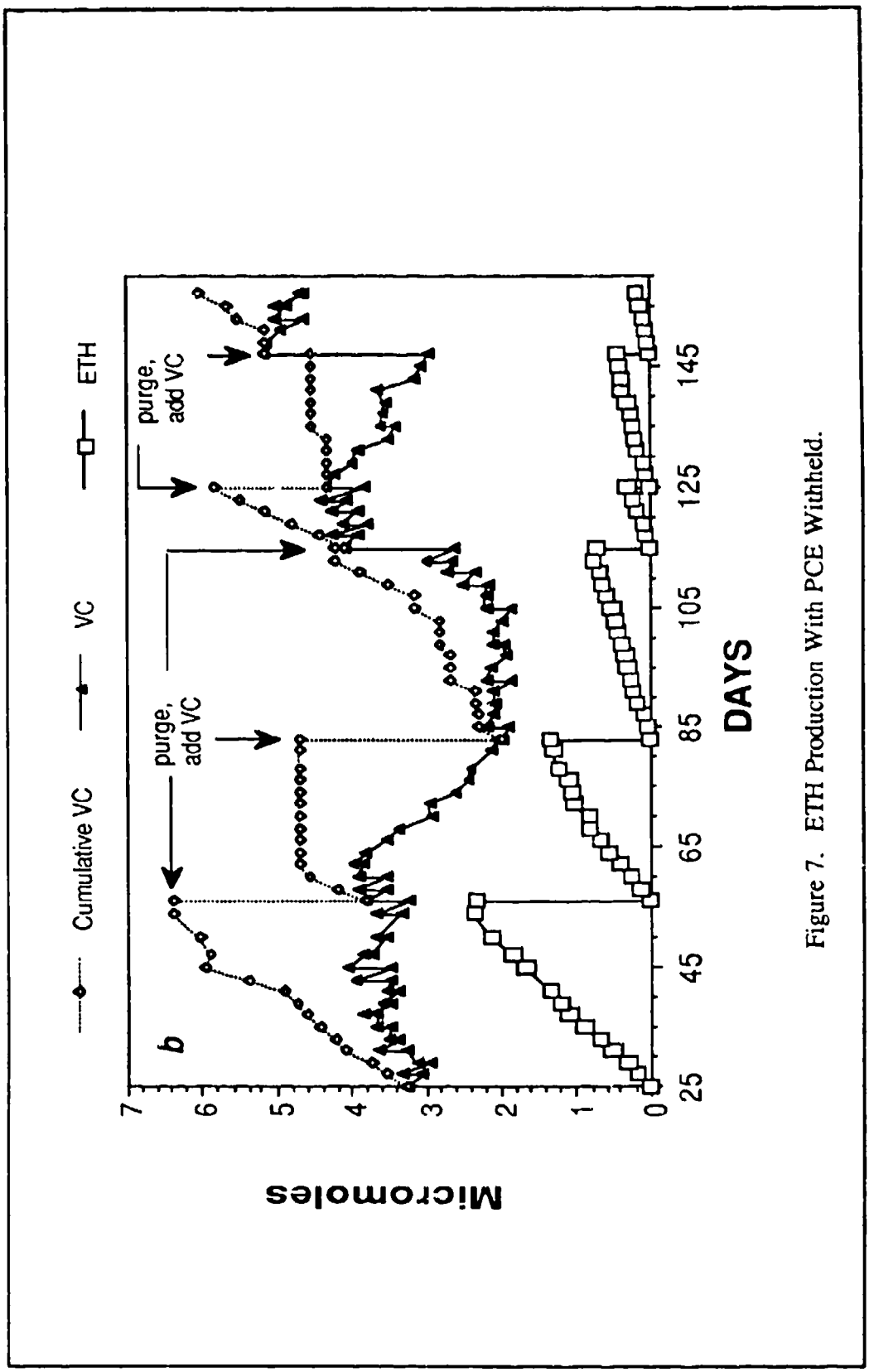



Figure 7. ETH Production With PCE Withheld.

For comparison, Figure 7 shows the progress of a VC-fed bottle. Ever-decreasing rates of ETH are obvious with each operating/purging cycle in the VC-fed bottles in which PCE additions were not resumed. ETH production rates are compared more easily in Figure 8. The upper graph indicates, in the absence of PCE, a decline in ETH production from 0.1 to 0.03 μmol per day. However, resumption of PCE additions after Day 65 seems linked to an increase in ETH production to 0.09 μmol per day on Day 124. ETH production increased after Day 85; routine PCE reduction had resumed at this time as shown in Figure 6. PCE was once again withheld and ETH production declined to less than 0.06 μmol per day by Day 145. By comparison, bottles in which PCE was permanently withheld (lower graph of Figure 8) exhibited a continuously declining ETH production rate from 0.1 μmol per day to less than 0.02 μmol per day.

It must be noted that although ETH production rates varied depending on treatments, $CH_4$ production remained similar in all bottles; near stoichiometric conversion of MeOH to $CH_4$ occurred routinely. This indicates that the observed decline in ETH production was not due to a decline in activity of methanogenic organisms in the culture.

## D. DISCUSSION

Observations indicate that once initiated, fairly constant conversion of PCE to ETH is sustainable by a MeOH-enriched, PCE-fed culture. When VC additions are substituted for PCE, an ever-decreasing ETH production rate was demonstrated. It is also clear that VC concentration alone does not determine ETH production rates; although VC was similar in all bottles, PCE-fed bottles produced and sustained ETH production while VC-fed bottles could not. This suggests that the presence of PCE (and /or TCE, DCEs) is (are) required to sustain VC dechlorination. Examination of Figure 3 indicates that appreciable ETH production did not occur until VC reached 2.5 μmol, a trend which was observed numerous times in other PCE-fed bottles. This lends support to the hypothesis that PCE *and* high levels of VC are required before ETH production is substantial.

The presence of PCE may be necessary to stimulate production of a nonspecific enzyme capable of mediating dechlorination. Recall that when PCE was withheld, and then reintroduced, a lag in dechlorination was noticed (Figure 6). Such a lag may mean that the organism is dechlorinating PCE for a purpose; for some reason, dechlorination of PCE may be worth the effort of enzyme synthesis. Indeed, the observation that PCE dechlorination seems to be inducible, and therefore serves a purpose, was noted previously (30).

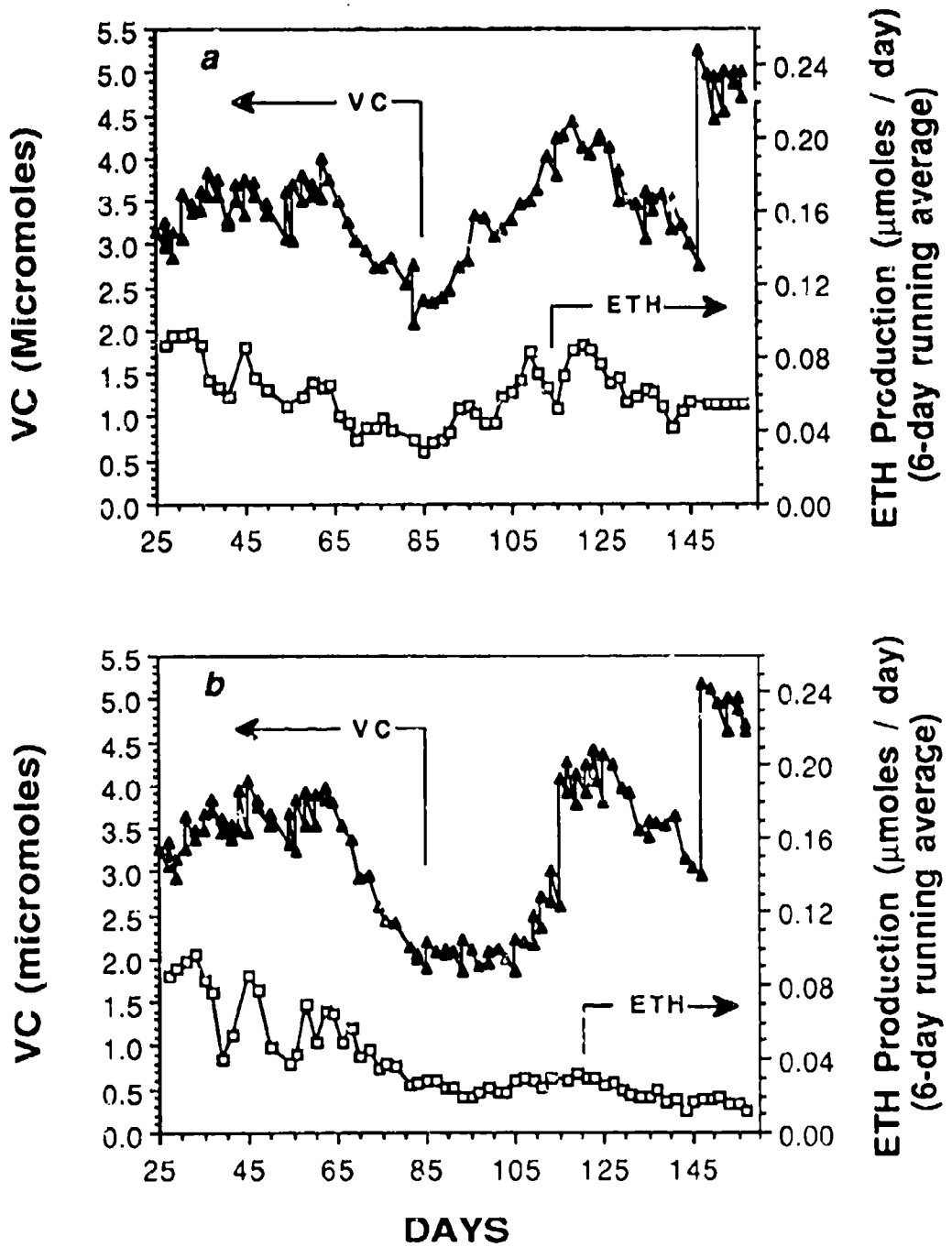



Figure 8. ETH Production Rates With (a) PCE Resumed and (b) PCE Withheld.

# SECTION V

# THE ROLE OF $H_2$ IN REDUCTIVE DECHLORINATION OF PCE

## A. CONTEXT

Glucose, HAc, formate, MeOH, and $H_2$ have each been shown capable of sustaining the reductive dechlorination of PCE (1), as have lactate, propionate, crotonate, butyrate, and ethanol (26). More exotic reductants, such as toluene (27) and dichloromethane (28), have also been observed to support dechlorination of PCE. The fact that such a wide variety of electron donors sustains dechlorination suggests that it may be the simplest — $H_2$ — which serves as the direct electron donor to the process; the other, more complex donors may merely serve as precursors for $H_2$ formation via fermentative metabolism (26).

In this Chapter, we present further characterization of our high-PCE enrichment culture — specifically, results from comparative studies with MeOH- and $H_2$-fed mixed anaerobic cultures amended with selective inhibitors (vancomycin, a eubacterial inhibitor; and 2-bromoethanesulfonic acid, an inhibitor of methanogenesis). Results provide additional evidence that $H_2$ is the direct electron donor for PCE reduction. We also report on the probable roles of major classes of organisms with respect to PCE dechlorination.

## B. PROCEDURES

### 1. Studies with $H_2$ as Electron Donor

The ability of $H_2$ to sustain dechlorination of PCE was investigated using four 160-mL serum bottles (100 mL liquid volume), each prepared by inoculating 90 mL basal medium with 10 mL of high-PCE/MeOH culture from the 6-liter reactor. $H_2$ (11.5 mL = 469 µmol) was added via a locking, gas-tight syringe every 2nd day to two bottles. For comparison purposes, the other two bottles received MeOH (5 mg from a 25 percent aqueous stock) every 2nd day as the electron donor. On a reducing-equivalent basis, $H_2$ (11.5 mL = 938 µeq) and MeOH (5 mg = 938 µeq) were provided in identical doses. The four bottles also received (every 2 days) neat PCE (55 µmol) and YE (0.2 mg from an anoxic, aqueous stock). Bottles were purged via cannula with 70 percent $N_2$/30 percent $CO_2$ prior to each feeding. By Day 6, each bottle had transformed its initial PCE dose; semicontinuous operation commenced at this point. Semicontinuous operation consisted of (every 2 days) gas-purging, wasting (via syringe) 10 mL mixed culture, and adding 10 mL basal medium. PCE, YE, and $H_2$ or MeOH were then added. Declining ETH production was observed in all bottles from Days 0 to 15 (see results); a micronutrient limitation was suspected; consequently, the YE dose was increased to 2 mg after Day 15. From Days 20 to 34,

culture wasting was suspended because ETH production had declined in all bottles; batch additions of PCE, YE, and $H_2$ or MeOH continued. After Day 34, ETH production had increased to over 20 µmoles per feeding in the MeOH bottles, and semi-continuous operation (every 2nd day) was resumed in all bottles.

### 2. Preparation of Filtered Culture Supernatant

For extended studies involving demonstration of $H_2$ as an electron donor, cultures were sometimes amended with filtered culture supernatant as a source of growth factors. Preparation of filtered culture supernatant included the following steps: (1) 600 mL PCE/MeOH culture was anaerobically transferred from the 6-liter high-PCE/MeOH stock culture to a 2-liter batch, anaerobic reactor; (2) a small volume (ca. 10 percent) of an aceticlastic methanogenic enrichment culture was added to consume HAc (normally present at high concentrations in the high-PCE/MeOH enrichment reactor; its removal was necessary to increase the precision of later results from electron-balance determinations); (3) when measurements indicated < 0.5 mM HAc remained (usually 5 to 8 days after inoculation), the culture was anaerobically transferred to centrifuge tubes while inside a glove box, centrifuged (4080 x *g*) outside the glove box, with the resulting centrate subsequently filtered (0.45 µm) inside the anaerobic glove box, to remove organisms; and (4) since the resulting filtrate obviously contained none of the black, iron-sulfide precipitate which normally helped maintain a low redox potential in our cultures, precipitate from basal medium was added to the filtrate.

### 3. Inhibitor Studies

The inhibitors vancomycin and BES were applied to high-PCE/MeOH and high-PCE/$H_2$ cultures to investigate the roles of acetogens and methanogens in PCE reduction. In an anaerobic glove box, PCE/MeOH inoculum from the high-PCE, 6-liter reactor was transferred to six 400-mL polypropylene bottles, which were then capped and centrifuged outside the glove box. The bottles were returned to the glove box, supernatant was wasted, and filtered culture supernatant was added to resuspend the pellet. (Treatment with filtered culture supernatant in this manner reduced the HAc concentration of the culture, facilitating the monitoring of subsequent, small changes in HAc levels during experimental runs.) Contents from all six were combined, and 100-mL aliquots of suspension were transferred to sixteen, 160-mL serum bottles. Teflon®-lined butyl-rubber septa and aluminum crimp caps were applied to the bottles, as usual.

The bottles were removed from the glove box and purged for 5 minutes with 70 percent $N_2$/30 percent $CO_2$. (The glove-box atmosphere was a $N_2$-$H_2$ composition; bottle purging was completed to replace the $N_2$-$H_2$ headspace with $N_2$-$CO_2$.) During purging, 10 mg (100 mg/liter) vancomycin was added to some bottles. After purging, 2 mg YE (20 mg/liter), and 156 µmol MeOH (50 mg/liter) or 469 µmol $H_2$ (11.5 mL) were delivered to the bottles. 55 µmol PCE

(91 mg/liter) was added to some bottles; others received no PCE, and served as controls. Later in the study, some bottles received 0.025 mmol BES (0.25 mM). Bottles were maintained in batch mode; every 2nd day, purging and additions of PCE, YE, MeOH, or $H_2$ were completed.

## C. RESULTS

### 1. $H_2$ as an Electron Donor for PCE Reduction

We first tested whether $H_2$ can serve as an electron donor for PCE dechlorination in short-term studies. PCE/MeOH cultures in 160-mL serum bottles were switched from MeOH to $H_2$ as the electron donor, and were incubated in a shaking water bath. The dose of $H_2$ employed was two-fold in excess of the stoichiometric requirement for complete dechlorination of PCE to ETH (as had been true of the MeOH dosages preceding the switch). As shown in Figure 9, such cultures immediately were able to use $H_2$ to convert PCE to ETH and small amounts of VC. Controls to which $H_2$ was not added did not dechlorinate PCE (data not presented).

These initial studies demonstrated that $H_2$ could serve as electron donor for PCE dechlorination — at least over the short term — in bottles started with 100 percent inoculum from the PCE/MeOH culture. We next prepared a more rigorous test of $H_2$, this time starting with 10 percent PCE/MeOH inoculum, and extending the study over 90 days to investigate possible nutritional deficiencies which might arise with use of $H_2$ in place of MeOH.

Figure 10 shows the performances of $H_2$-fed and MeOH-fed bottles. VC and ETH productions by the $H_2$-fed and MeOH-fed bottles were similar for the first 10 days. ETH production in the $H_2$-fed bottles diminished to insignificant levels by Day 10. VC production continued; however, residual PCE was routinely detected. By contrast, the MeOH-fed bottles transformed each PCE dose (after Day 20) to VC and ETH. TCE was rarely detected and insignificant levels of DCEs were produced. As the study progressed, the $H_2$-fed bottles experienced a progressively diminishing ability to dechlorinate. Figure 10 shows the dramatic increase of TCE beginning after Day 35. It seemed that $H_2$ could serve as an electron donor, but could not sustain PCE reduction.

The inability to sustain dechlorination to VC and ETH could have been due to a lack of growth factors in the $H_2$-fed bottles — factors which may have been produced in the MeOH-fed systems. Serum bottles were prepared with filtered culture supernatant from MeOH enrichments to test this hypothesis. The 6-liter PCE/MeOH culture provided seed organisms for this experiment. PCE/MeOH culture (100 mL) was centrifuged and the resultant supernatant was removed. The inoculum pellet was then suspended in sufficient filtered culture supernatant to restore the 100-mL liquid volume. A batch mode of operation was adopted to avoid dilution of the presumed growth

factors contributed by the supernatant diluent. Every 2nd day, after headspace analysis, a purging and feeding procedure was completed as before.

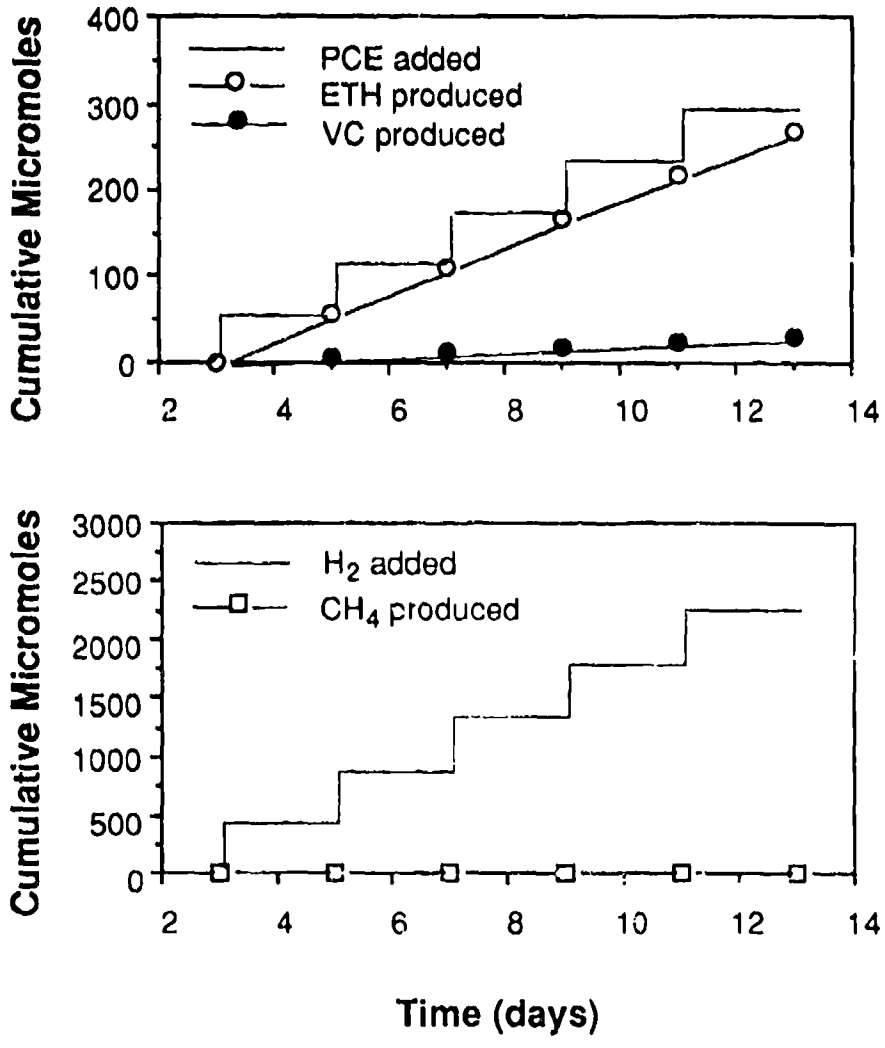



Figure 9. Short-Term Results Demonstrating the Ability of $H_2$ to Support Dechlorination of 55 µmol Repetitive Additions of PCE to ETH; Bottles Were 160-mL in Total Volume, Containing 100 mL of Liquid.

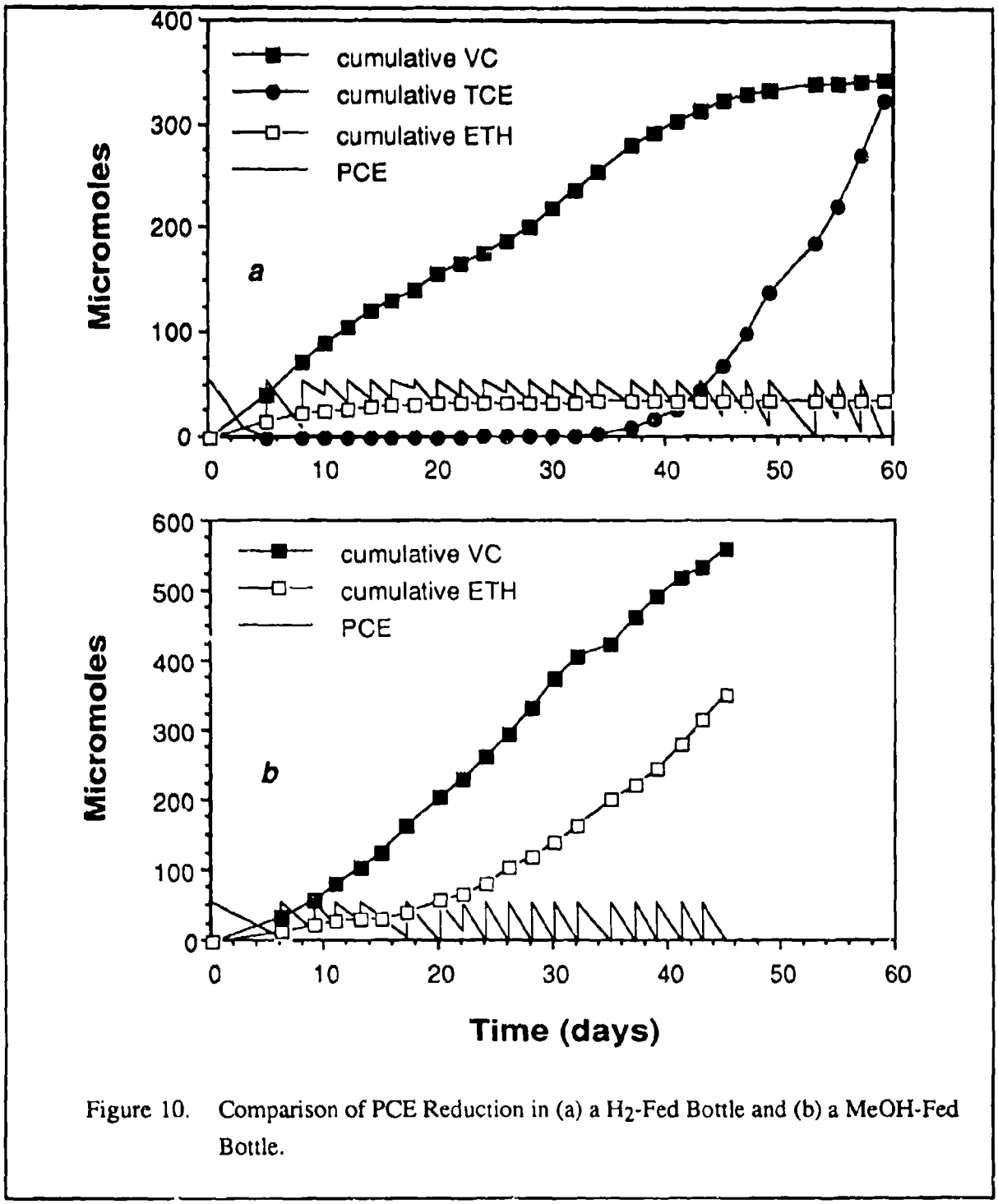



Figure 10. Comparison of PCE Reduction in (a) a $H_2$-Fed Bottle and (b) a MeOH-Fed Bottle.

Results (not shown) indicated that $H_2$-fed systems could sustain dechlorination if elutriated with filtered culture supernatant approximately every 30 days (29). To demonstrate that $H_2$ (as opposed to filtered culture supernatant) was supplying reducing equivalents for dechlorination of PCE to ETH, $H_2$ was withheld from the bottle on an every-other-feeding basis from Day 96 onward. The purging procedure and PCE and YE additions were completed during every feeding. Incubation intervals with $H_2$ resulted in conversion of all added PCE to VC and ETH, with more ETH formed than VC. During intervals in which $H_2$ was withheld, 20 to 30 μmoles residual PCE was detected, and ETH production was insignificant.

### 2. Application of Inhibitors to MeOH- and $H_2$-Fed Cultures

Inhibitors were applied to the PCE/MeOH or PCE/$H_2$ cultures to investigate the roles of different microbial populations in PCE reduction. Vancomycin is an inhibitor of cell wall synthesis in eubacteria to which methanogens are usually insensitive (36). Although $CH_4$ production was slight in the high-PCE culture, this alone does not rule out a role for methanogens in PCE reduction. BES — considered a selective inhibitor of methyl-coenzyme-M reductase, the enzyme which catalyzes the final step in methanogenesis (37) — was therefore used to inhibit methanogenesis in the PCE culture.

The performance of a MeOH-fed bottle receiving no inhibitors is shown in Figure 11. VC and ETH productions were steady up to Day 14; afterwards, VC declined slightly as ETH production increased. Insignificant levels of TCE and DCEs sometimes occurred, and residual PCE was never detected. MeOH metabolism and resultant products are shown in Figure 11, as well as the summation of dechlorination products, to allow comparison to the usage of MeOH, the presumed electron donor. Residual MeOH was never detected, so MeOH consumed is essentially equal to MeOH added. Acetogenesis accounted for almost 60 percent of electron-donor use, and reduced products from PCE dechlorination represented over 40 percent. $CH_4$ production was insignificant. These results are similar to an earlier-reported distribution of MeOH use (2).

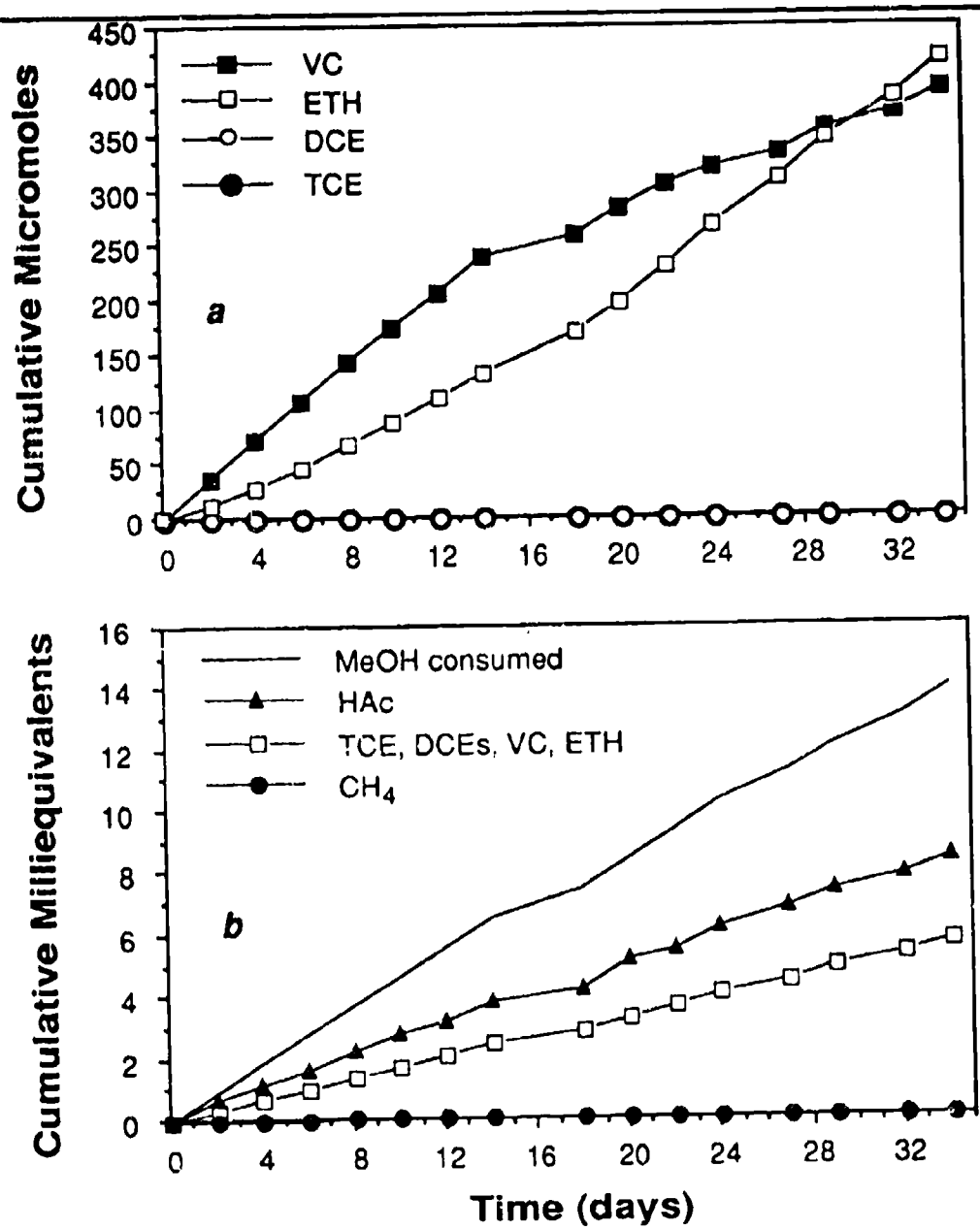



Figure 11. (a) PCE Reduction and (b) MeOH Metabolism in a MeOH-Fed Culture.

PCE reduction and $H_2$ metabolism by a bottle receiving no inhibitors are shown in Figure 12. PCE reduction to VC and ETH occurred during every incubation interval. Insignificant levels of DCEs sometimes occurred; TCE or residual PCE was never detected. VC production was greater than that of ETH up to Day 15; VC and ETH productions were similar thereafter. (The superior dechlorination in this $H_2$-fed system, compared with that of the system depicted in Figure 10, is doubtless due to the ten-fold greater inoculum level employed — and the consequent ten-fold greater supply of suspected growth factors from the PCE/MeOH inoculum.) $H_2$ metabolism and resultant products are shown in Figure 12. Added $H_2$ was used completely during each incubation period. Acetogenesis accounted for 54 percent of electron-donor use, and reduced products from PCE dechlorination represented nearly 45 percent. $CH_4$ production was slight; PCE apparently inhibited methanogenesis from $H_2$-$CO_2$, as well as from HAc.

Vancomycin was added to two MeOH-fed and two $H_2$-fed bottles to observe potential effects on PCE reduction. Results from the vancomycin-amended, MeOH-fed bottle are shown in Figure 13. Vancomycin had a profound effect on PCE reduction. Essentially equal amounts of VC and ETH were produced up to Day 8. Thereafter, ETH production ceased and VC production declined. Increasing amounts of residual PCE were observed beginning on Day 10. Figure 13 shows only slight HAc production after Day 8, thus demonstrating the inhibitory effect of vancomycin on acetogens (a smaller vertical scale is used than that of Figure 11 for clarity). From Days 8 to 14, nearly all MeOH utilization can be accounted for in the products of PCE reduction. However, only slight increases in dechlorination products are evident after Day 14. $CH_4$ production from MeOH became significant between Days 14 to 18, when we allowed a 4-day interval between successive PCE doses. Residual PCE was probably insufficient to suppress methanogenesis over this prolonged interval. This confirms that vancomycin, at the dose employed, did not severely inhibit methanogenesis. Note that $CH_4$ production decreased over ensuing, 2-day intervals between feedings. Residual MeOH was routinely detected. The MeOH level was adjusted to 156 µmol per bottle (50 mg/liter) during each feeding to validate the comparison between vancomycin-amended bottles and those with no vancomycin.

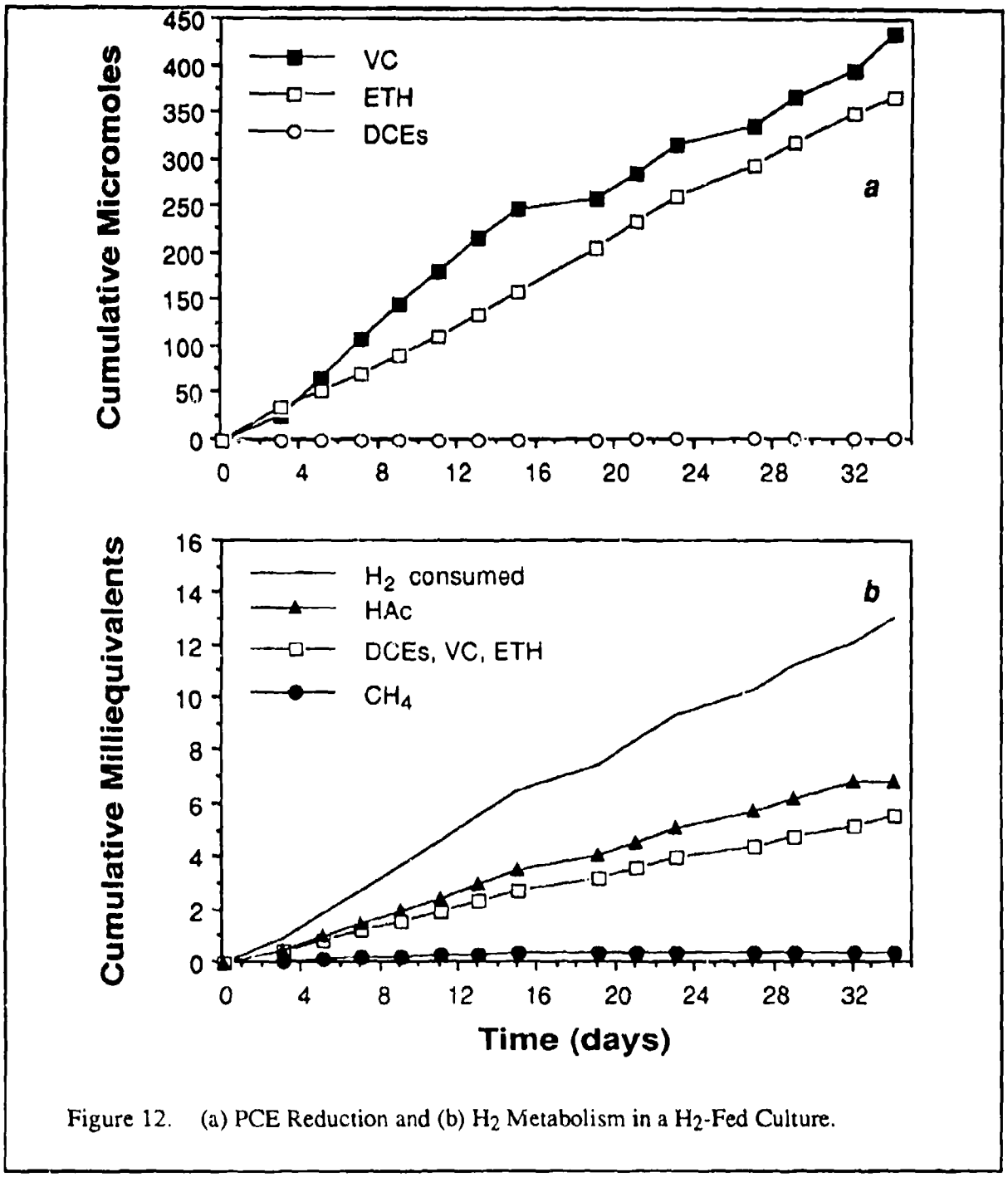



Figure 12. (a) PCE Reduction and (b) $H_2$ Metabolism in a $H_2$-Fed Culture.

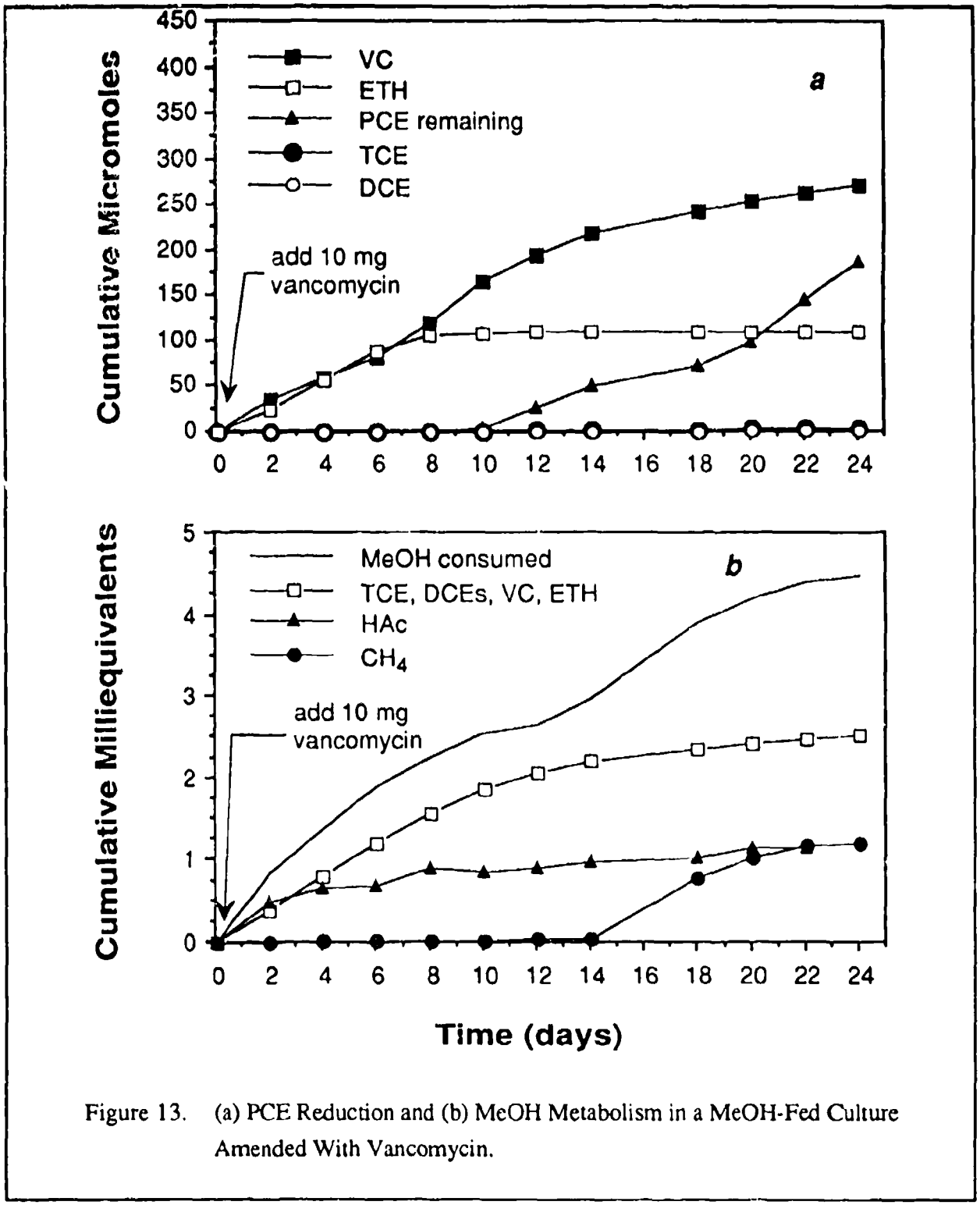



Figure 13. (a) PCE Reduction and (b) MeOH Metabolism in a MeOH-Fed Culture Amended With Vancomycin.

PCE reduction and $H_2$ metabolism by a vancomycin-amended bottle are shown in Figure 14. PCE reduction by this $H_2$ enrichment continued in the presence of vancomycin. VC production was similar to that of $H_2$-fed bottles with no vancomycin (Figure 12) throughout the study. ETH production was similar up to Day 16, but declined to low levels by Day 27. TCE and DCEs were detected on Day 34; residual PCE was never observed. Inhibition of acetogenesis by vancomycin was apparent from the low HAc production shown in Figure 14 (a smaller vertical scale is used than that of Figure 12 for clarity). Since it is possible that the low HAc detected in vancomycin-amended bottles was due to consumption by aceticlastic methanogens rather than inhibition of acetogenesis, bottles containing $H_2$ and vancomycin were later dosed with BES and did not accumulate HAc (results not shown), making it unlikely that aceticlastic methanogens were consuming HAc being produced in these cultures. Products of PCE reduction accounted for nearly 60 percent of $H_2$ use in vancomycin-amended bottles. $CH_4$ production represented a significant fraction of the remainder of $H_2$ use, primarily from Days 0 to 20. $CH_4$ production declined after Day 19, however, PCE reduction products (primarily VC) continued to increase. Residual $H_2$ was routinely detected in the vancomycin-amended bottles. $H_2$ did not accumulate since it was removed during purging. All $H_2$-fed bottles began each incubation interval with an equivalent $H_2$ dose. Thus, the dechlorinating performance of the vancomycin treated PCE/$H_2$ cultures was similar to cultures without vancomycin, while acetogenesis was strongly inhibited, and methanogenesis was stimulated.

The decline of ETH production and detection of TCE and DCEs indicated that the $H_2$-fed bottles with vancomycin were slowly losing the capability to dechlorinate PCE. An elutriation procedure with filtered culture supernatant (as described earlier) plus vancomycin was twice completed (Days 36 and 64) on the vancomycin-amended culture with the expectation that growth factors would stimulate PCE reduction. Batch feeding and purging were maintained every 2nd day throughout this study. After the first elutriation, from Days 36 to 64, VC production was steady and accounted for most of the dechlorinated PCE. Only low levels of ETH were detected throughout this cycle. Small amounts of TCE and DCEs were routinely detected. Improved dechlorination was evident after the second elutriation on Day 64. Increased ETH production occurred up to Day 79; TCE and DCEs increased thereafter. VC production remained consistent throughout each cycle. Elutriation successfully restored ETH production, although to a lesser rate than observed from Days 0 to 15.

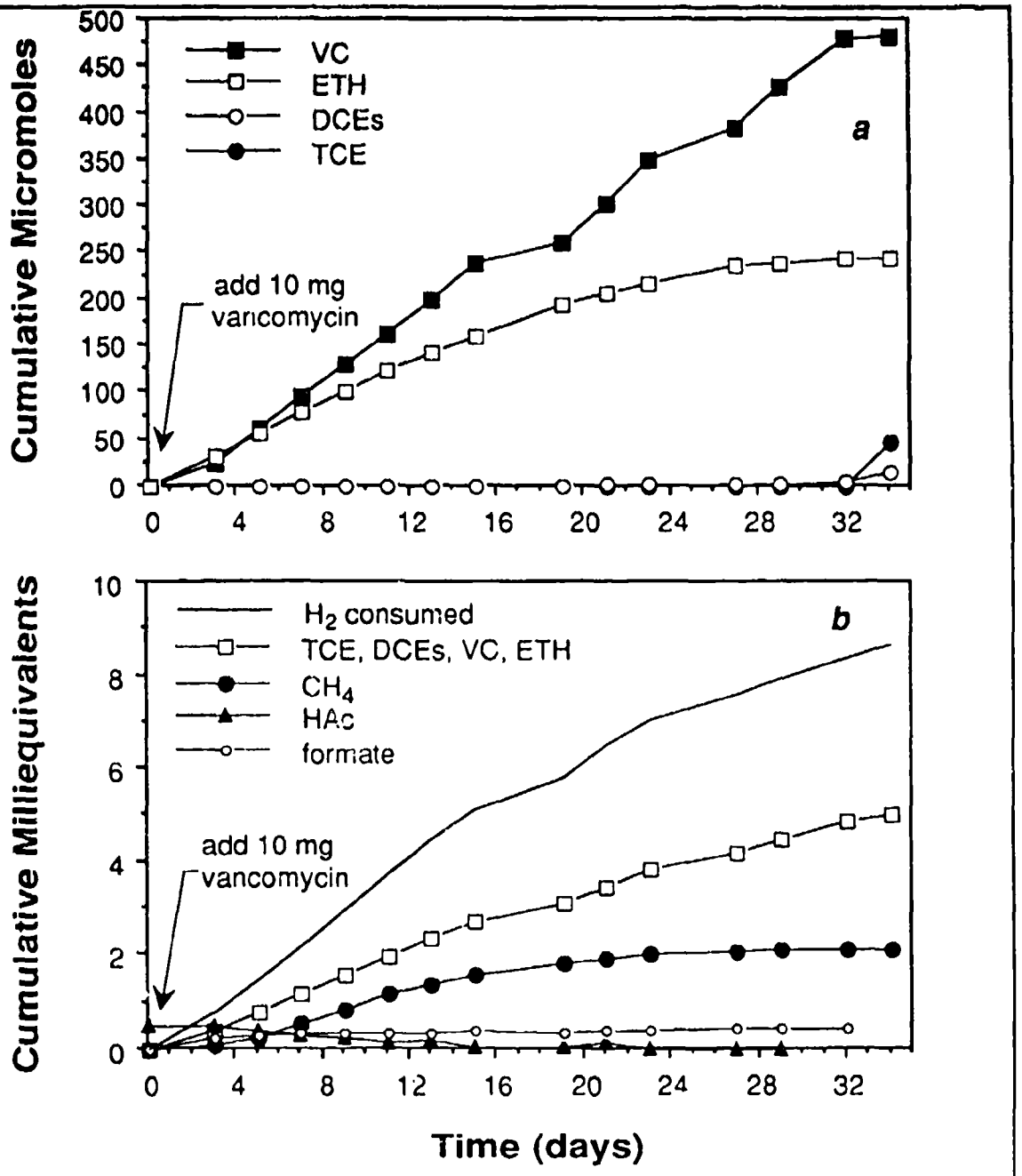



Figure 14. (a) PCE Reduction and (b) $H_2$ Metabolism in a $H_2$-Fed Culture Amended With Vancomycin.

The effects of BES on dechlorination of PCE in MeOH- and $H_2$-fed systems were examined. Earlier results (Figures 11 and 12) indicated $CH_4$ production was negligible in PCE-fed MeOH and $H_2$ enrichments, suggesting that methanogens play no significant role in dechlorination. Surprisingly, 0.25 mM BES inhibited PCE dechlorination in a MeOH-fed system (data not shown), with no apparent effect on acetogenesis. Up to Day 27, this bottle performed similarly to its replicate (Figure 11), as expected. However, after BES addition on Day 27, VC and ETH productions were slight. Accumulations of TCE and DCEs were observed, but residual PCE was not. Similar inhibition of dechlorination was observed with $H_2$-fed cultures.

## D. DISCUSSION

$H_2$ served as the electron donor in the reductive dechlorination of PCE to VC and ETH for periods up to 40 days; however, sustained dechlorination for more extended periods required the addition of filtered culture supernatant from a presumably more diverse, MeOH-fed system. This suggests a nutritional dependency of $H_2$-using dechlorinators upon the activities of other organisms.

MeOH/PCE and $H_2$/PCE systems without vancomycin or BES performed similarly with respect to electron-donor use; HAc production accounted for a majority of reducing equivalents with the remainder going to PCE reduction. The predominance of acetogens in the inoculum was also evident from the MeOH- and $H_2$-fed bottles in which PCE was withheld (data not shown) — MeOH-using methanogens were apparently in much lower numbers than MeOH-using acetogens; HAc-using methanogens were virtually absent.

Vancomycin effectively inhibited acetogenesis in MeOH- and $H_2$-fed bottles of all types. Vancomycin is a eubacterial peptidoglycan synthesis inhibitor, is bacteriocidal, and is more effective against Gram-positive than Gram-negative eubacteria (36, 38). It is unlikely that vancomycin directly inhibited acetogenesis in the cultures, but rather the inhibition was due to the bacteriocidal effects of vancomycin on acetogens, most of which are Gram-positive (39). The major MeOH-utilizing acetogen detected in the culture was a Gram-positive coccus which was present in numbers exceeding $10^7$/mL (40).

The effects of vancomycin on PCE dechlorination were mixed. PCE reduction by MeOH enrichments was inhibited within a week, similar to acetogenesis. In contrast to MeOH-fed bottles, dechlorination by $H_2$-fed bottles continued well after inhibition of acetogenesis, and could be sustained for long periods of time by supplementation with filtered culture medium, similar to $H_2$-fed bottles lacking vancomycin. The difference in response to vancomycin by $H_2$- and MeOH-fed bottles may be explained if $H_2$ was the actual electron donor for dechlorination in both systems. It is feasible that acetogens in the MeOH-fed culture were the major users of MeOH and served two

purposes to the PCE dechlorinators — production of reducing equivalents in the form of $H_2$, and production of unknown nutritional factors apparently required by $H_2$-using dechlorinators. Reductive dechlorination is considerably more thermodynamically favorable than acetogenesis (2), and use of acetogen-produced $H_2$ by other organisms carrying out more thermodynamically favorable reactions has been observed previously (41). Moreover, interspecies nutritional co-dependencies are well-known among anaerobes. That the PCE dechlorinators are resistant to 100 mg/L vancomycin, suggests that they are resistant eubacteria, perhaps Gram-negative, or methanogenic archaebacteria.

The methanogenesis inhibitor BES inhibited dechlorination beyond TCE in MeOH- and $H_2$-fed systems. This provides circumstantial evidence for the involvement of methanogens in reductive dechlorination — in the absence of appreciable methanogenesis itself. Fathepure et al. (21, 22, 23) reported transformation of PCE to TCE by pure methanogenic cultures, but observed a stoichiometric relationship between methanogenesis and dechlorination, and noted that BES inhibited dechlorination. They proposed that electrons transferred during methanogenesis are diverted to PCE by a reduced electron carrier involved in $CH_4$ production. For us to conclude a dechlorinating role for methanogens in our cultures, based solely upon observations that BES inhibits dechlorination, presumes that BES-induced inhibition is specific to methanogens due to its structural similarity to CoM, which is unique to methanogens. However, BES is a brominated alkane and bears structural similarity to PCE and its reduced products. It is conceivable that BES would inhibit reductive dechlorination, regardless of whether or not the dechlorinating organism was a methanogen.

Taken together, our vancomycin and BES results with MeOH/PCE- and $H_2$/PCE-fed systems support a hypothesis that the dechlorinating organisms were $H_2$-users, possibly (though not necessarily) methanogens. Sustained dechlorination in the presence of vancomycin (which demonstrably inhibited acetogenesis), suggests that acetogens were probably not the dechlorinators.

Figure 15 shows the hypothetical roles of methanogens and acetogens in our high-PCE/MeOH enrichments; high levels of PCE inhibited methanogenesis from MeOH, HAc, and $H_2$. Vancomycin inhibited acetogens, thereby stopping the source of reducing equivalents for PCE reduction. We suggest that dechlorination continued in $H_2$-fed systems amended with vancomycin, since the proposed electron donor, $H_2$, was always available. In this study, PCE-reduction products accounted for 60 percent of $H_2$ use in vancomycin-amended bottles.

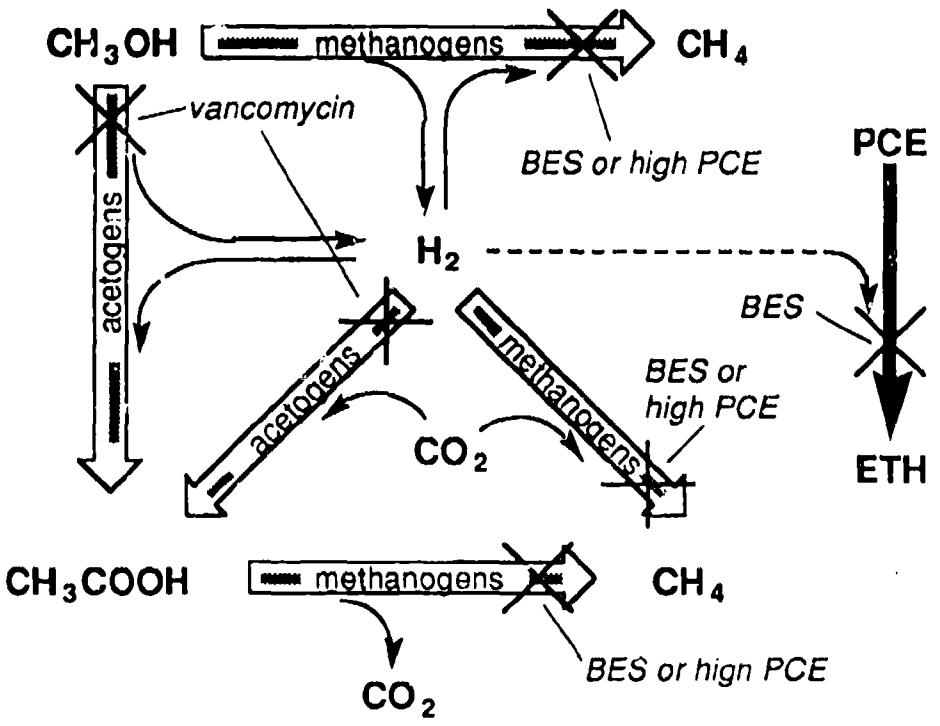



Figure 15. Hypothetical Roles of Methanogens and Acetogens in $H_2$- and MeOH-Fed PCE-Dechlorinating Systems.

If borne out by subsequent studies, our hypothesis that $H_2$ is the actual electron donor for PCE dechlorination has practical consequences for bioremediation. It explains why a wide variety of electron donors has been observed to support PCE dechlorination in mixed-culture systems: glucose, HAc, formate, and MeOH (1); sucrose (33, 34); lactate, propionate, crotonate, butyrate, and ethanol (26); and even co-contaminants such as toluene (27) or dichloromethane (28). All likely produce at least a small pool of $H_2$; and a small pool would be sufficient to transform ppb-levels of PCE. For bioremediation of relatively high PCE levels, electron donors should be selected which cause production of a large $H_2$ pool — or methods may be explored which would employ $H_2$ directly. One caveat is the apparent nutritional dependency we observed of $H_2$-using dechlorinators in our culture upon other organisms. If dechlorinating organisms in other systems have the same or similar nutritional requirements, the required nutritional factor(s) must be identified and abiotically supplied, or it may be impossible to exclusively target electron donor solely to the dechlorinators.

# SECTION VI

# PERFORMANCE OF THE HIGH-PCE/MeOH ENRICHMENT CULTURE AT LOW PCE CONCENTRATIONS

## A. CONTEXT

Through previous research conducted at Cornell University's Environmental Engineering Laboratory, an anaerobic, MeOH-fed PCE enrichment culture was developed that proved capable of dechlorinating high concentrations of PCE (550 μM) to VC and ETH (2). This "high-PCE/MeOH" enrichment culture was developed from a methanogenic system which originally sustained dechlorination of a low level of PCE (3.5 μM) (1); the major product of dechlorination was VC, and conversion of VC to ETH was not appreciable in this low-PCE culture. As this methanogenic culture was exposed to increasing doses of PCE, methanogenesis became inhibited, and the "high-PCE" culture evolved which demonstrated an ability to sustain dechlorination of high levels of PCE at relatively low levels of MeOH. MeOH was supplied as an electron donor at a level which was approximately twice the amount needed for the complete transformation of PCE to ETH. The efficient use of the MeOH equivalents supplied for reductive dechlorination in this culture appeared to result from the inhibition of methanogenesis, which was apparently caused by exposure of methanogenic bacteria to high concentrations of PCE.

Since many contaminated groundwaters contain concentrations of PCE which are significantly lower than 550 μM, it was desired to undertake studies which explored the capability of this high PCE culture to sustain dechlorination of lower levels of PCE. Could dechlorination be sustained at reasonable MeOH/PCE ratios at noninhibitory levels of PCE; or would methanogens rise in dominance and consume an ever-increasing fraction of the supplied MeOH?

## B. PROCEDURES

This first experiment studied the ability of the high-PCE enrichment culture to sustain dechlorination of varying levels of PCE and ratios of MeOH to PCE. Four feeding strategies were analyzed, and each condition was replicated in two bottles. Table 2 summarizes the conditions employed.

TABLE 2. LEVELS OF SUBSTRATE EMPLOYED IN BOTTLE-EXPERIMENT I

| Bottle Set | Nominal PCE (mg/L) | MeOH (mg/L) | MeOH:PCE (equiv. basis) | Yeast Extract (mg/L) |
|---|---|---|---|---|
| 1 | 91 | 50 | 2:1 | 20 |
| 2 | 9.1 | 50 | 20:1 | 20 |
| 3 | 9.1 | 5 | 2:1 | 2 |
| 4 | 9.1 | 25 | 10:1 | 10 |

Bottle Set 1 was maintained in a manner similar to that of the high-PCE culture in the 6-liter inoculum reservoir. A nominal concentration of 91 mg/L was achieved by injecting 9.1 mg (55 µmol) neat PCE into the 160-mL bottles with a 10-µL syringe. MeOH was added to Bottle Set 1 in neat form with a similar 10-µL syringe to achieve a concentration of 50 mg/L (156 µmol), an amount which corresponded to approximately a 2:1 MeOH:PCE ratio (equivalent basis). YE was delivered to the bottles via syringe at the same concentration fed to the high-PCE enrichment culture. This amount was 20 mg/L, or 2 mg YE per bottle. Preparation of the YE stock solution was described previously in Section III. The remainder of the bottles employed PCE at a level which was an order of magnitude less than that maintained in Bottle Set 1. This amount, 5.5 µmol PCE per bottle (or nominally 9.1 mg/L), was also delivered in neat form using a 1-µL syringe. MeOH was delivered in neat form as well, according to Table 2 above, and 1-µL or 10-µL syringes were employed as appropriate. YE was delivered at an amount which equaled the same ratio of YE:MeOH (mass basis) employed in the 6-liter culture (0.416 mg YE:mg MeOH), using either 10- or 100-µL syringes.

The eight cultures were prepared as follows: all bottles were purged initially to create anoxic conditions as described in Section III. The high-PCE culture provided the necessary inoculum. A volume of 100 mL of 100 percent inoculum was delivered anaerobically to each of the bottles with a Unispense II pump (Wheaton industries). The eight bottles were immediately fed after inoculation and thereafter maintained similarly to the 6-liter culture for two 4-day cycles at a high level of PCE (nominally 91 mg/L) and MeOH (50 mg/L), to assure that transfers had gone well and that all bottles performed comparably at start-up. On the 2nd and 4th day, each bottle was fed appropriate amounts of PCE, MeOH and YE. On the 4th day of the cycle, the bottles were purged for 5 minutes with an oxygen-scrubbed mixture of 70 percent $N_2$/30 percent $CO_2$ to remove inhibitory levels of ETH and VC. Also, an aliquot of 10 mL was removed from each bottle on every 4th day, and replaced with 10 mL fresh basal salts medium via syringe; therefore,

the HRT of the bottles was maintained at 40 days. After purging, wasting and feeding, the bottles were provided with fresh septa, and were fed appropriate amounts of MeOH, PCE, and YE. Measurements of pH made on the wasted 10 mL of culture on every 4th day. The bottles were maintained at high PCE levels for two cycles of 4 days before the feeding protocol outlined in Table 2 was followed. Appropriate headspace measurements of PCE, TCE, DCEs, VC, ETH and $CH_4$ and liquid measurements of HAc were made during the course of the experiment, as described in Section III. The bottles were maintained in an inverted position in an orbital shaker bath which was isothermally controlled at 35°C. The duration of the bottle experiment varied with each bottle set, and ranged from 34 to 42 days.

## C. RESULTS

The culture replicates behaved similarly, and the general trend of each culture is described as follows: Figures 16 and 17 indicate the performance of Bottle Set 1, which was similar to the behavior of the 6-liter high PCE culture in that methanogenesis was not a major activity, and after every 2-day interval, nearly all of the PCE delivered to the cultures was transformed to approximately 30 percent VC and 70 percent ETH, as indicated by the distribution of ethenes in the Figures. Measurements every 2nd day indicate product levels resulting from the feeding which occurred 2 days prior; measurements taken every 4th day represent product formation resulting from two previous feedings. After product measurement on the 4th day of the cycle, all volatiles were purged and the cycle repeated itself; this pattern is evident in the "saw-tooth" plots in subsequent figures. After every 2 days, there was little or no TCE or DCEs remaining in the cultures. Inhibition of methanogenesis was evident, caused by the high level of PCE employed in the cultures. In Figures 16 and 17, an accounting of the products formed during each 2-day feeding interval on an electron equivalent basis indicated that dechlorination and methanogenesis accounted for approximately 47 percent and 7 percent, respectively, of the electron equivalents provided to the cultures by the addition of MeOH. HAc production accounted for the remaining 46 percent of the electron equivalents supplied, since HAc levels remained steady at approximately 600 mg/L (0.01 M); therefore the removal of 10 mL of culture medium (which contained 800 μeq HAc) every 4 days must have been balanced by the production of 800 μeq HAc every 4 days. This production of HAc would account for the difference of approximately 400 μeq between the addition of MeOH and the sum of ETH and $CH_4$ productions shown. Throughout the experiment, VC, ETH and $CH_4$ formation remained relatively steady. Bottle 1B indicated a small amount of residual PCE on Days 4 and 8, but thereafter converted all of the added PCE into ETH and VC. Circumneutral pH was measured throughout the experiment.

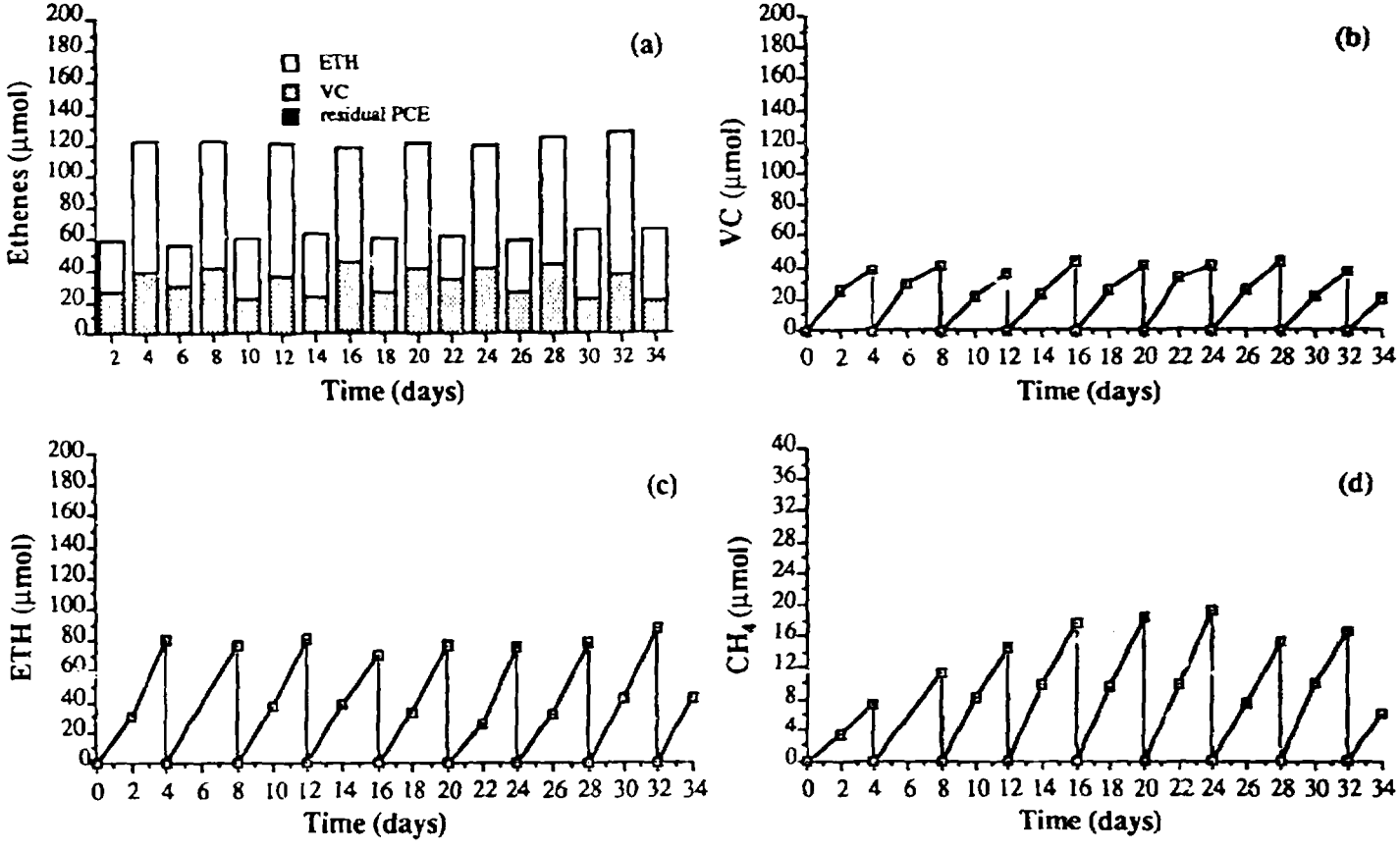



Figure 16. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 1A.

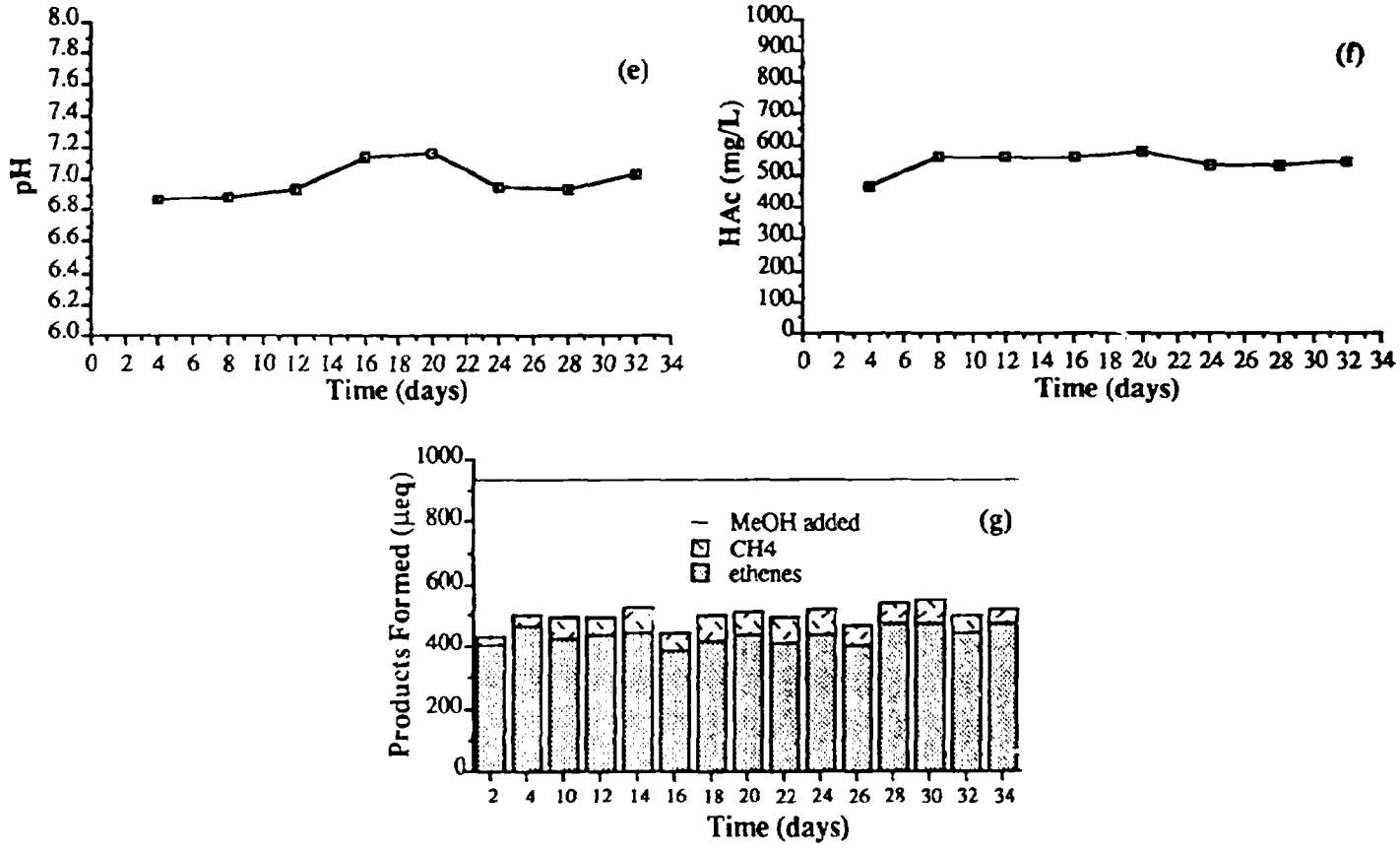



Figure 16. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 1A (Concluded).

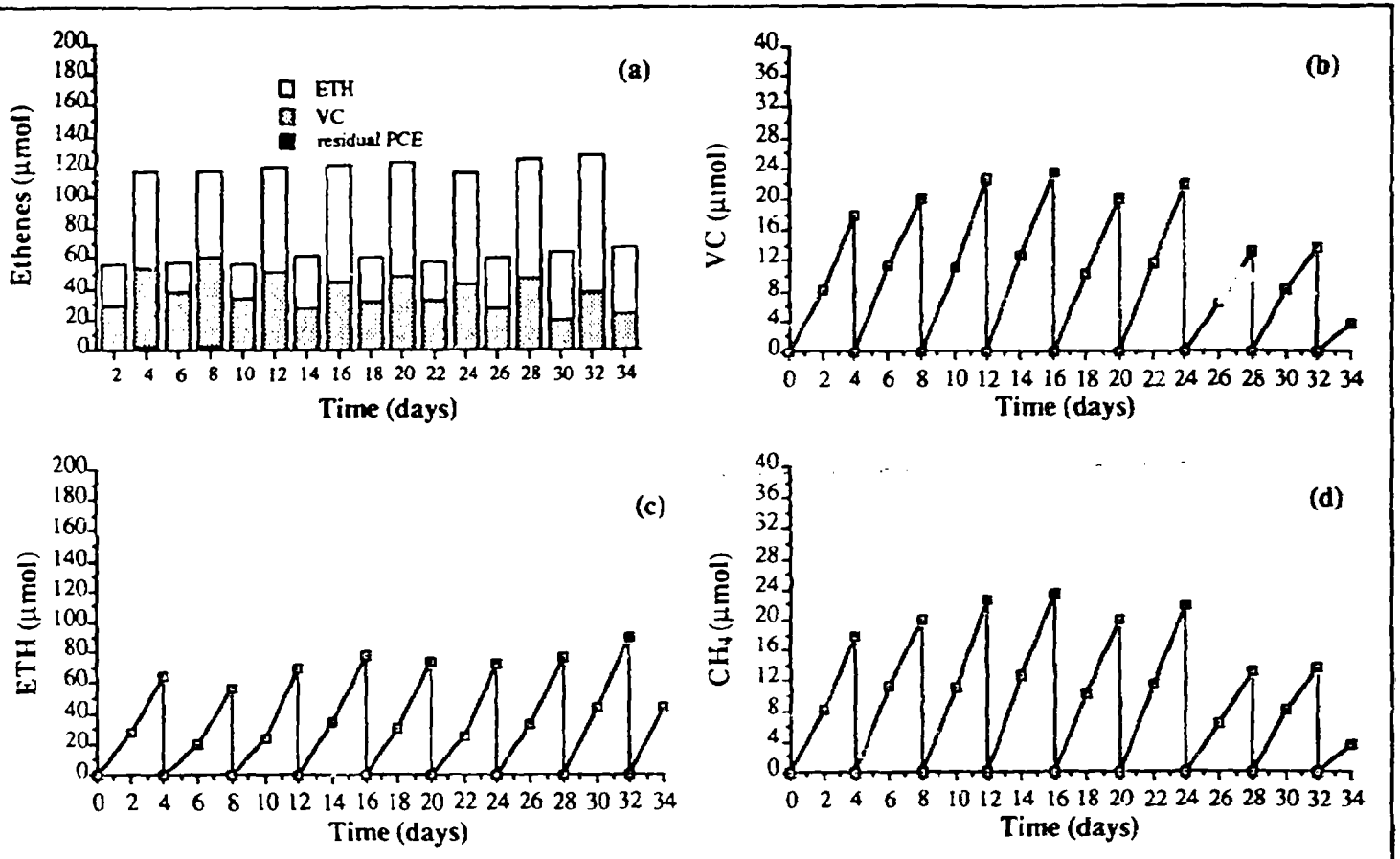



Figure 17. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) $CH_4$ Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 1B.

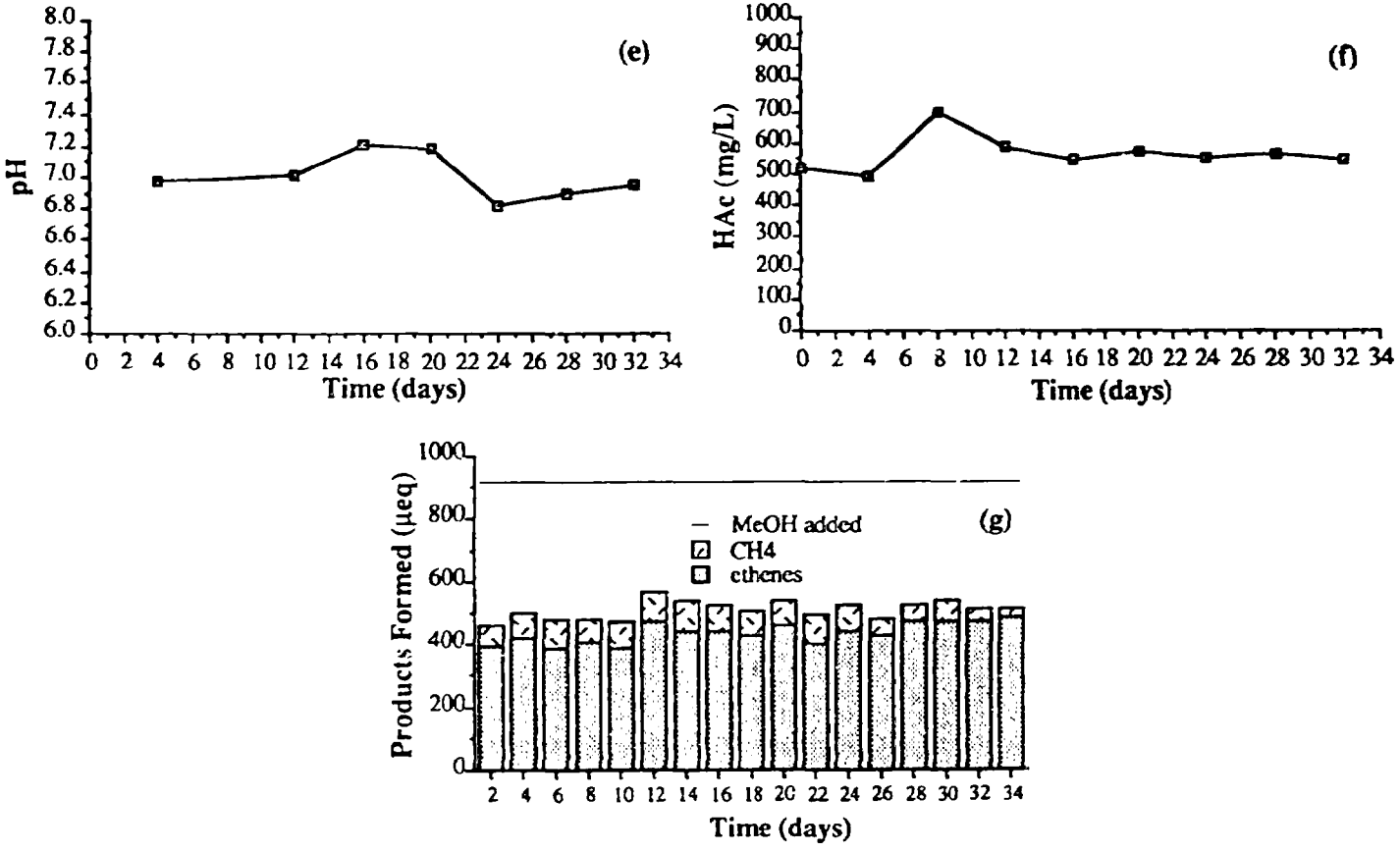



Figure 17. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 1B (Concluded)

Bottle Set 2 (Figures 18 and 19) was fed one order of magnitude less PCE than Bottle Set 1 and was provided with MeOH at a 20:1 ratio, on an electron-equivalent basis. Since methanogenesis was no longer inhibited by a high level of PCE, and also a relatively large amount of MeOH was provided, the culture gradually shifted from one in which dechlorination was a major activity to one in which methanogenesis proliferated. $CH_4$ formation surged after Day 12, and from this point $CH_4$ accounted for most of the electron equivalents provided by the donor. The measured $CH_4$ yield after this time was nearly equal to the theoretical yield according to $CH_3OH \rightarrow {}^1/_4\ CO_2 + {}^3/_4\ CH_4 + {}^1/_2\ H_2O$.

After Day 12, ETH production declined, and the levels of VC in the bottles increased for the remainder of the experiment. Although Bottle 2A indicated no residual PCE after each 2-day feeding interval, Bottle 2B was not able to transform all of the PCE into reduced products during three 2-day intervals throughout the course of the study. It is apparent on Day 26 that this culture was adversely affected, as nearly half of the supplied PCE remained as residual, but on the next feeding day, the culture appeared to recover, as no residual PCE was left. The large amount of $CH_4$ produced between Days 26-28 indicated that the MeOH which was fed on the first part of the 4-day cycle was not used until the latter 2 days of the cycle. It is assumed that acetogenesis and biomass formation account for the imbalance of equivalents of reduced products presented in Figures 18 and 19. A high background level of HAc was present in the cultures initially since the inoculum for the cultures originated from the high-PCE level culture which produced quantities of HAc much larger than levels of HAc produced by a predominantly methanogenic culture. Hence, accurate measurements of incremental HAc production were not feasible in the transformed methanogenic cultures of Bottle Set 2. Circumneutral pH was measured throughout the experiment.

In Bottle Set 3, a feeding protocol incorporating a low level of PCE, along with MeOH provided at a 2:1 ratio (electron-equivalent basis), also resulted in a gradual transformation of the cultures to predominantly methanogenic ones, as indicated in Figures 20 and 21 by a surge in a $CH_4$ formation after Day 12, a simultaneous decline in ETH production, a rise followed by a decline in VC levels, and residual PCE remaining after 2-day intervals. As with Bottle Set 2, actual $CH_4$ formation was nearly equal to the theoretical yield after Day 16. Again, the large background of HAc from the inoculum did not allow for accurate measurements of HAc production over time. For most of the time during the experiment, the amount of equivalents of reduced products formed exceeded the amount of equivalents provided by the MeOH; this imbalance might have been caused by the addition of reducing equivalents which were supplied by the digestion of organic material contained in the YE solution, or by endogenous sources. [In previous studies conducted by DiStefano (29), YE was found to provide 42 microequivalents of reductant per milligram of YE supplied.]

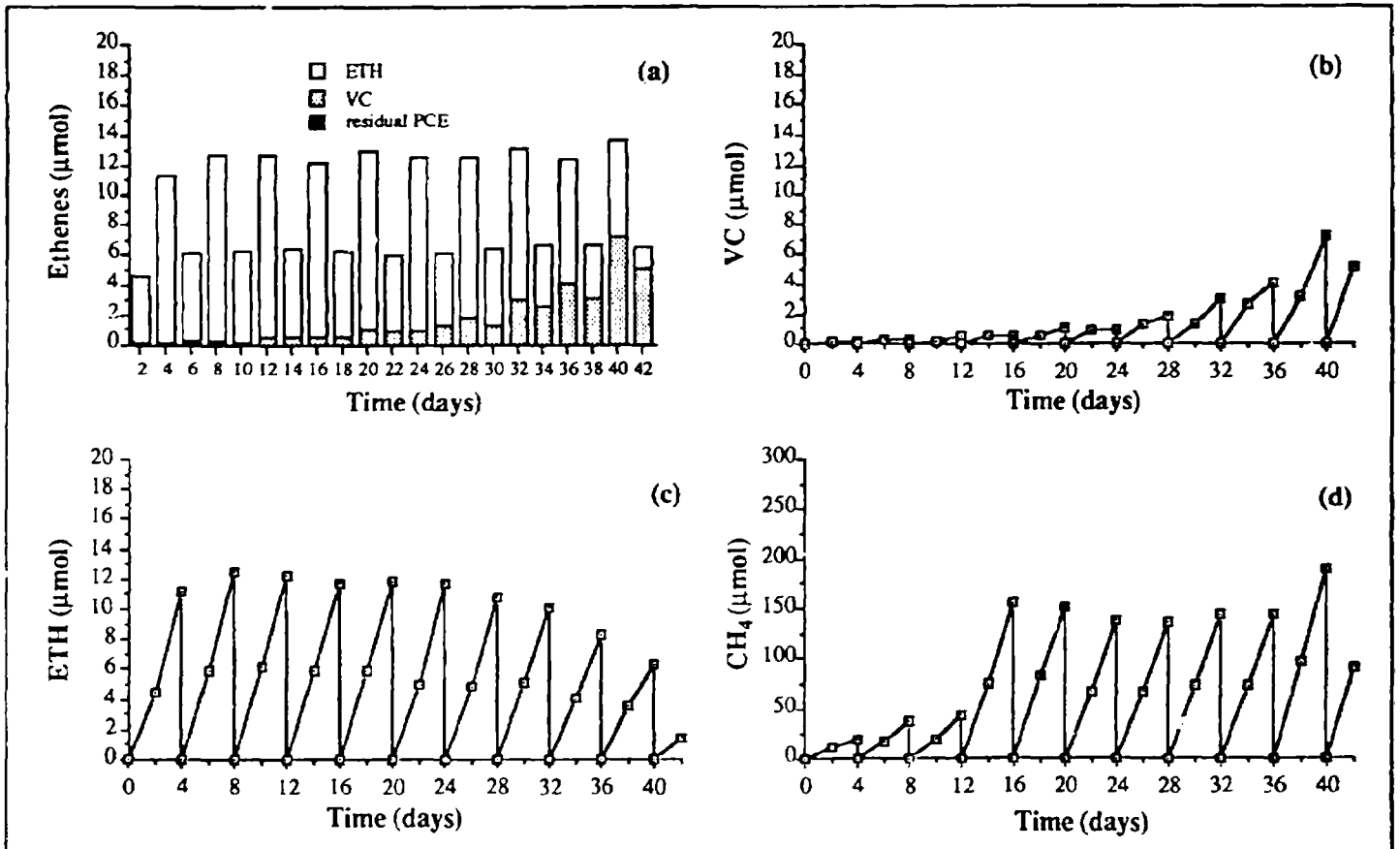



Figure 18. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) $CH_4$ Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 2A.

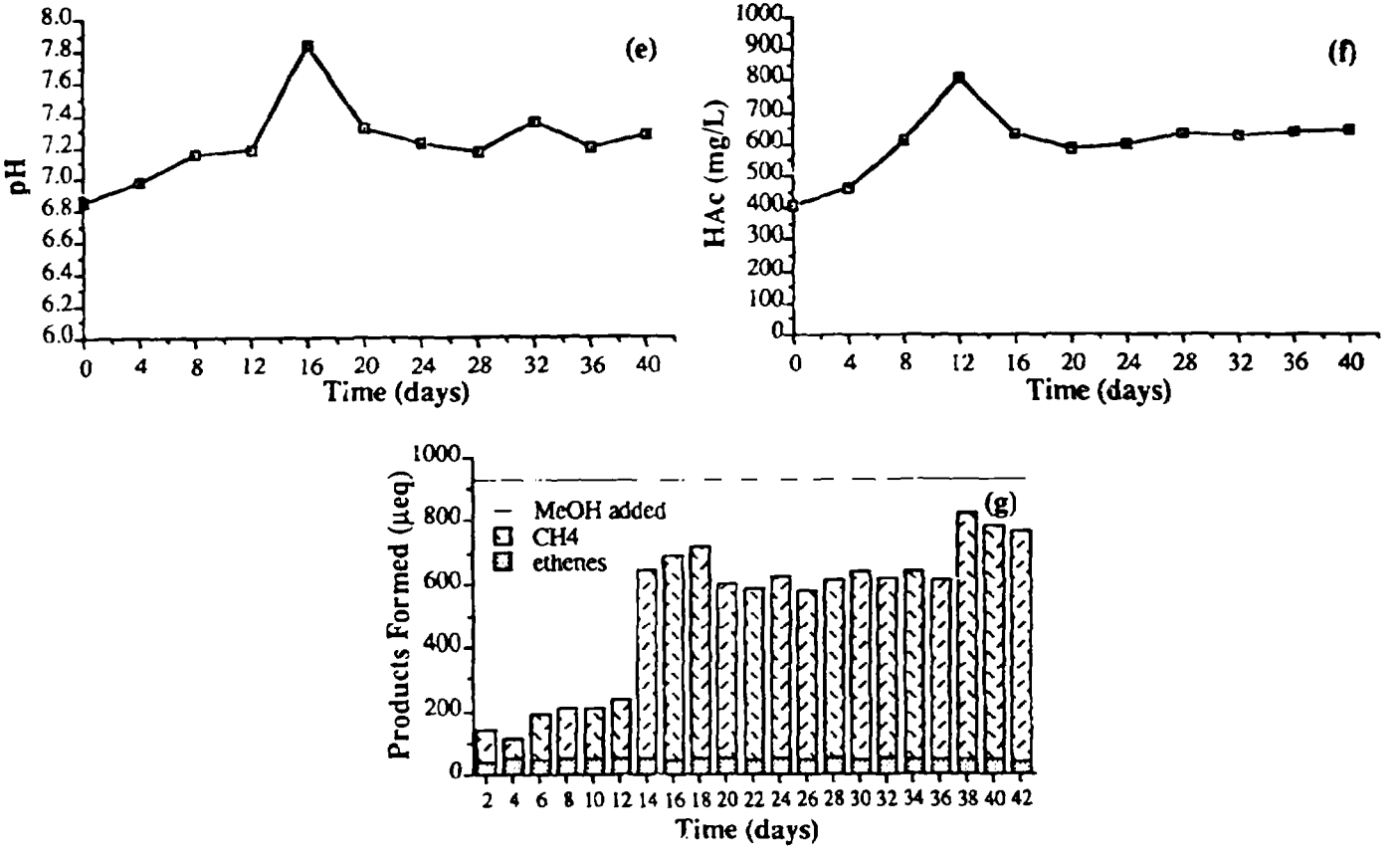



Figure 18. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 2A (Concluded).

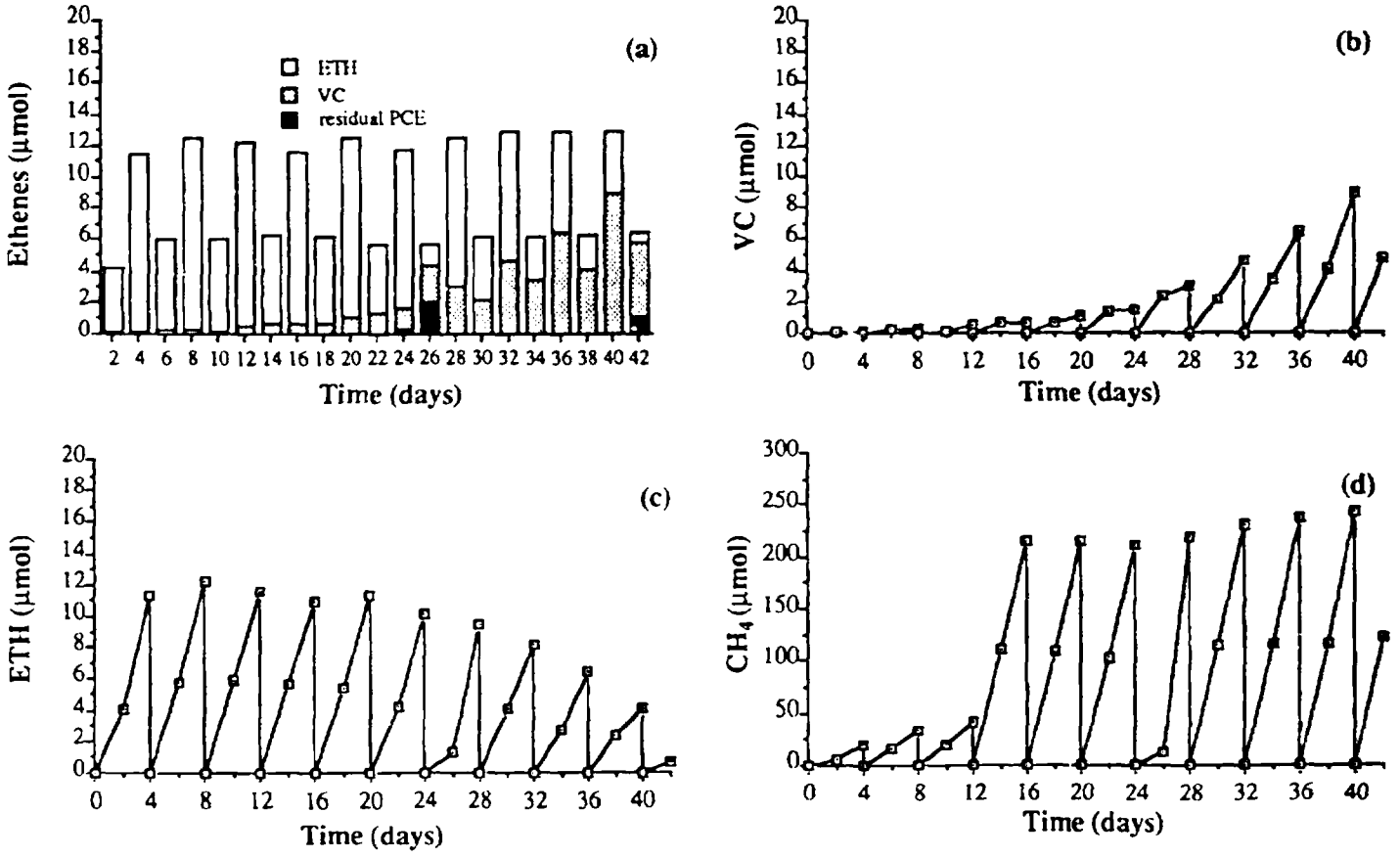



Figure 19. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) $CH_4$ Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 2B.

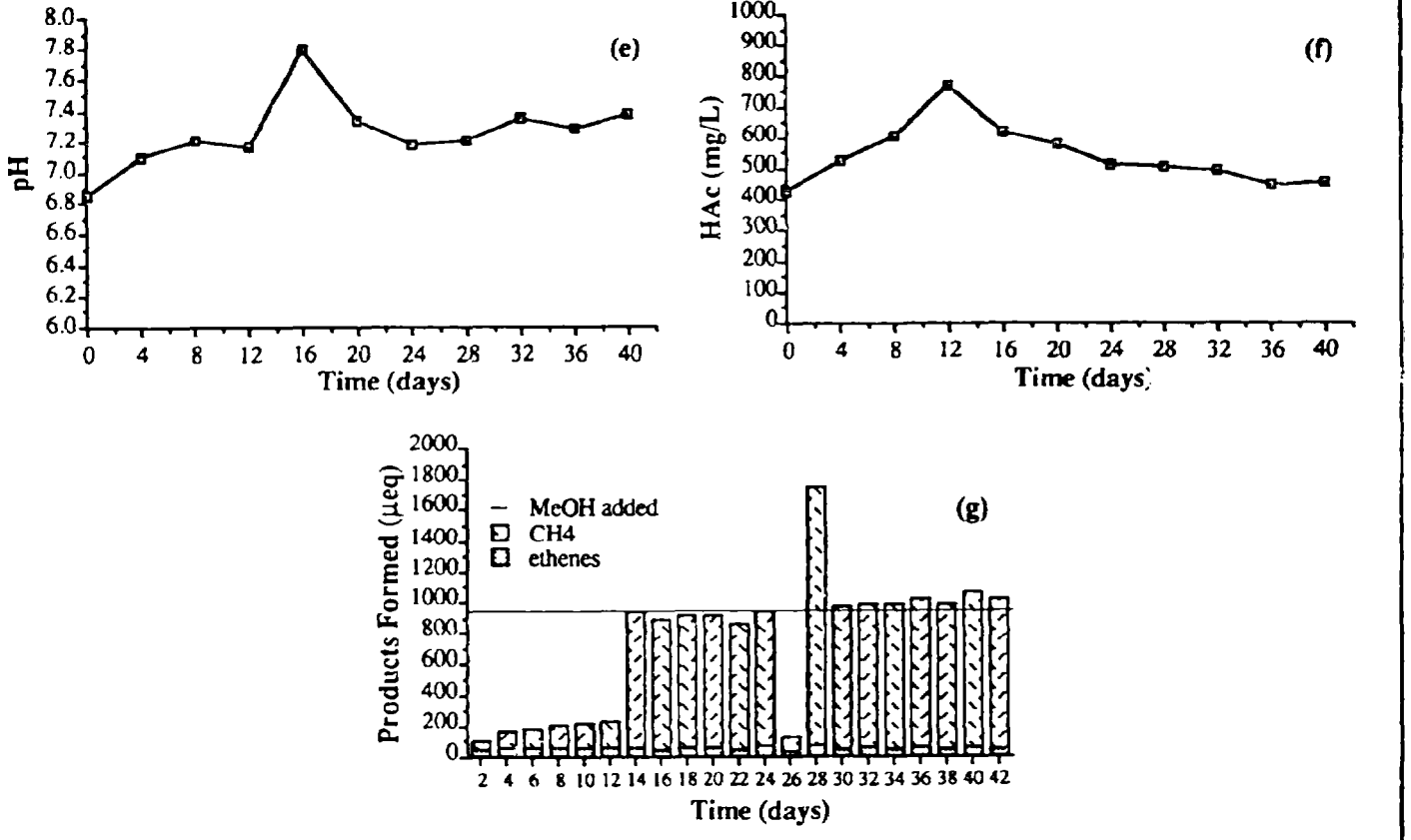



Figure 19. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 2B (Concluded).

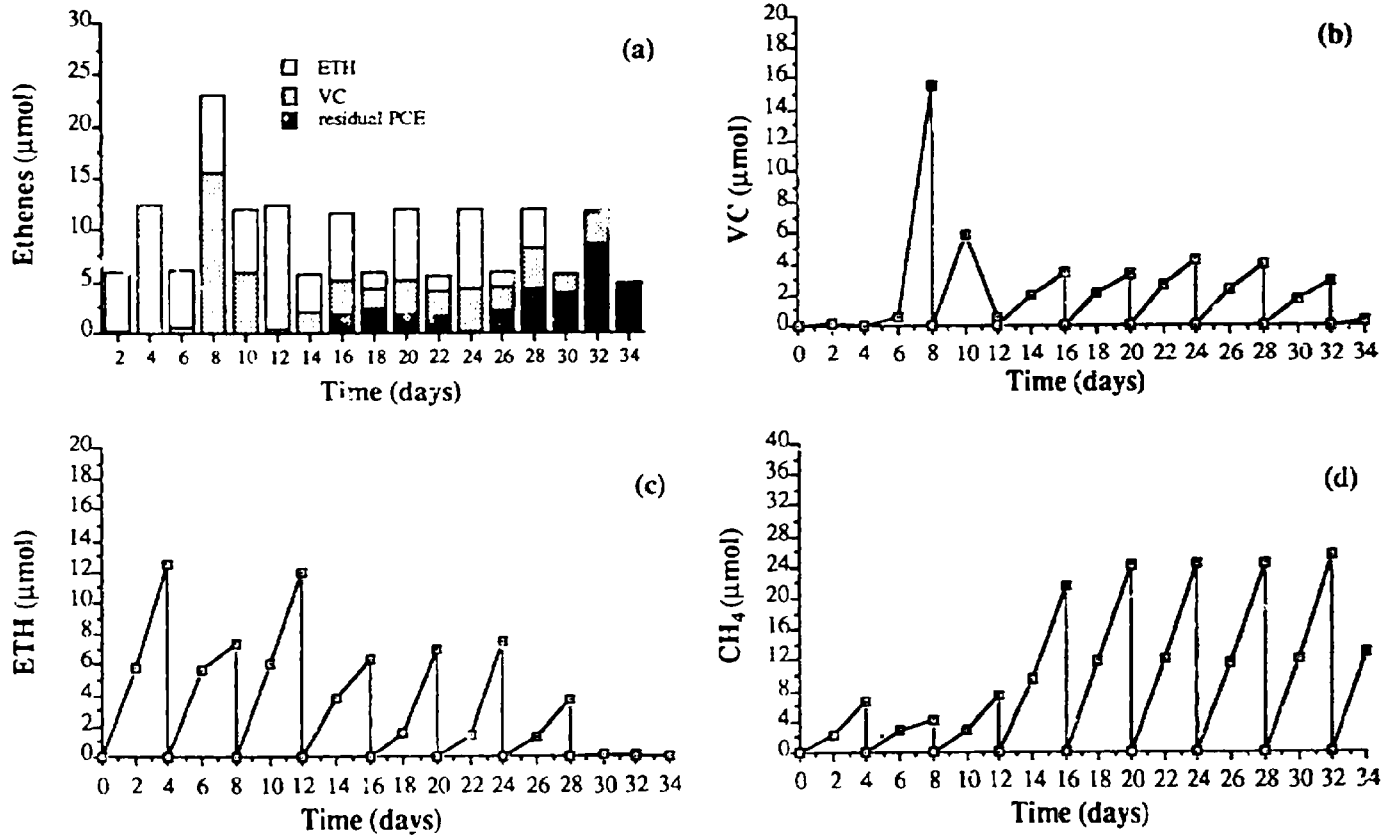



Figure 20. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 3A.

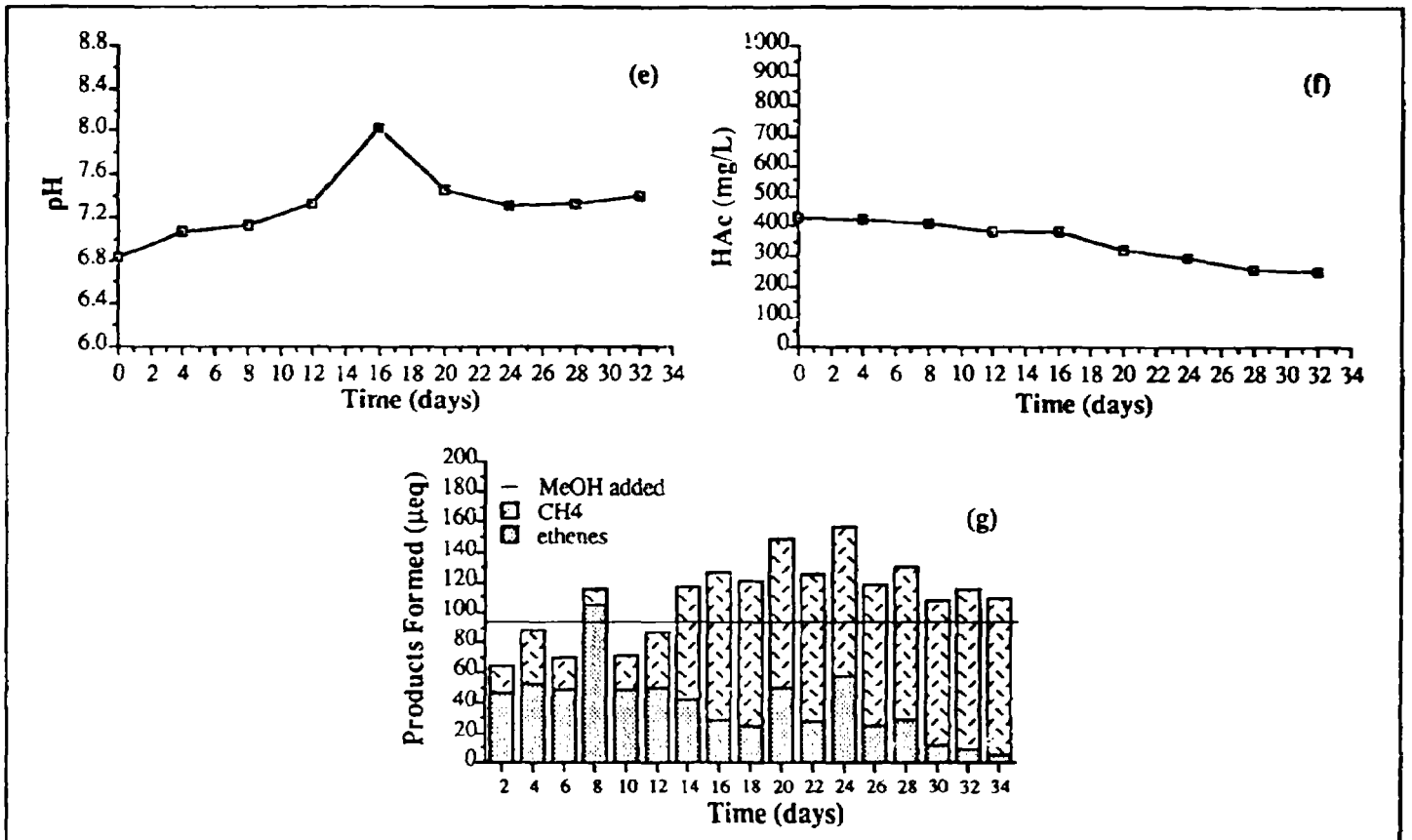



Figure 20. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 3A (Concluded).

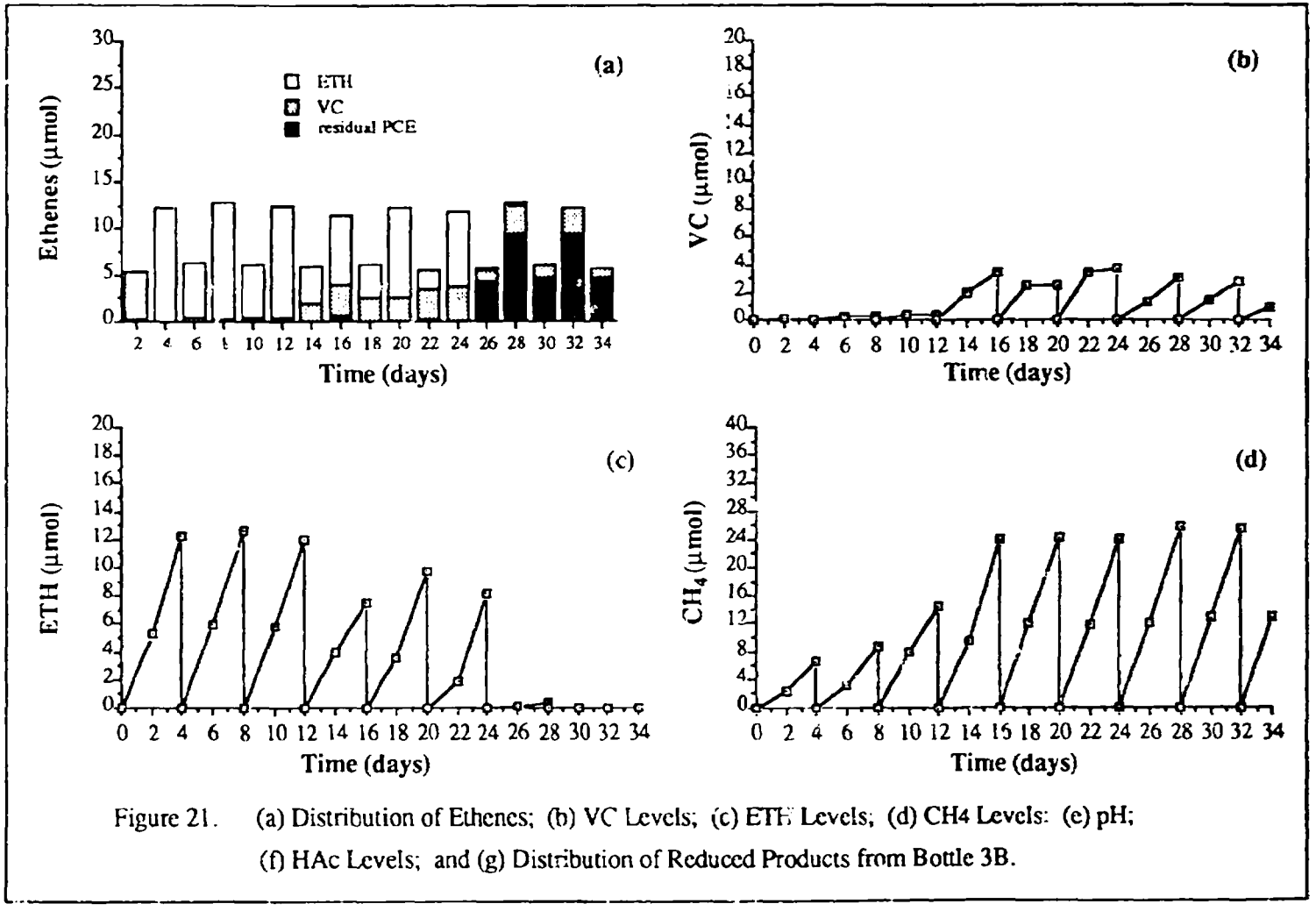



Figure 21. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels: (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 3B.

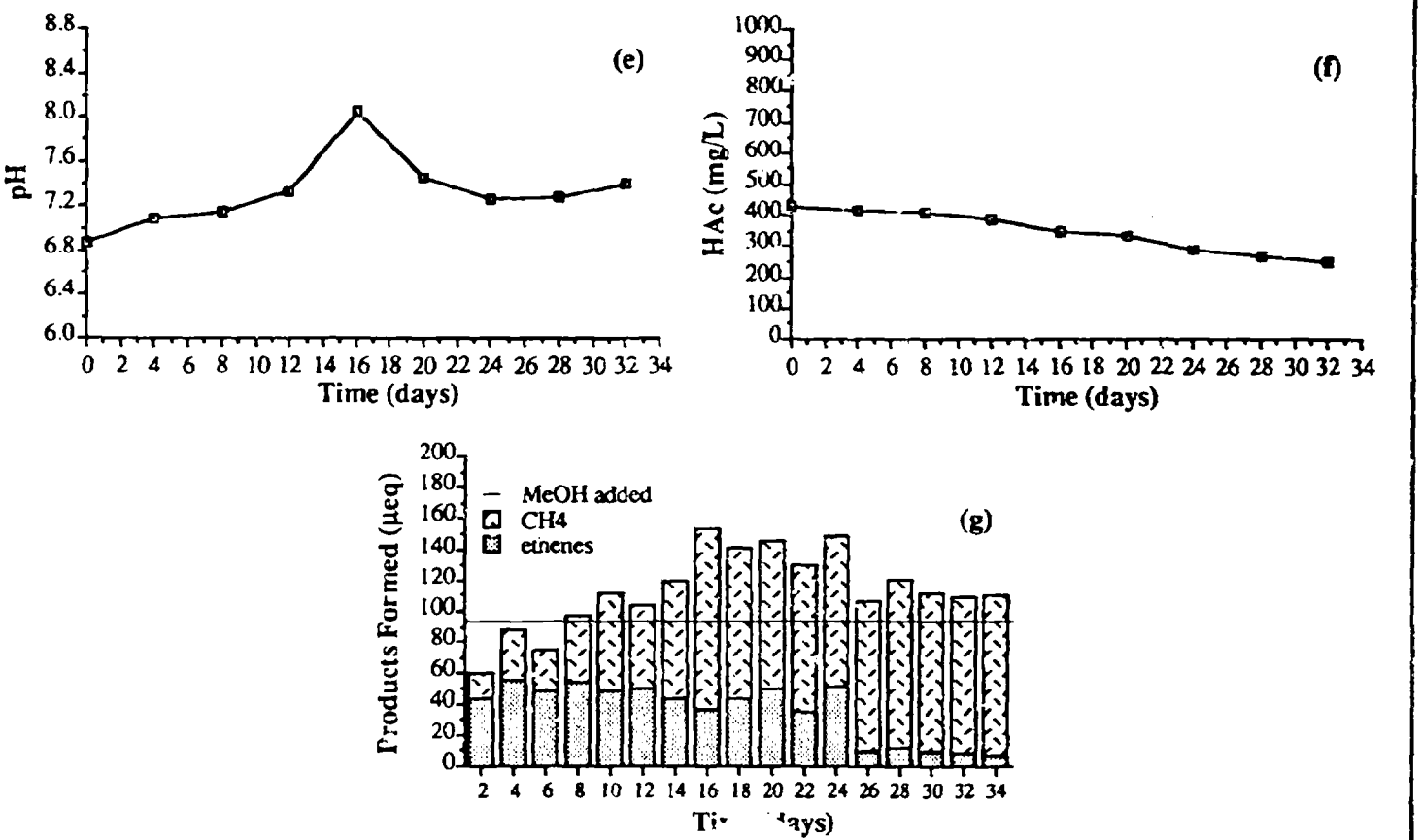



Figure 21. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 3B (Concluded).

On Day 16, a high pH reading of about 8.1 was observed in both replicates of Bottle Set 3, possibly causing the residual PCE indicated on this day. This mishap was probably due to an improper purging mix of $N_2/CO_2$ on Day 12. The pH during the remainder of the study maintained circumneutral values. A final anomaly is to be noted; on Day 6 of Bottle 3A, an apparent incorrect feeding of PCE resulted in a high accounting of ethenes on Day 8.

Bottle Set 4, which incorporated a low level of PCE and an amount of MeOH which corresponded to a 10:1 electron equivalent ratio of MeOH:PCE, behaved in a manner similar to that of Bottle Set 2 (20:1 MeOH:PCE ratio) in that gradually ETH production declined and VC levels rose over time, as indicated in Figures 22 and 23; methanogenesis did not surge, however, until Day 24, after which the actual $CH_4$ yield equaled the theoretical yield, and the culture was transformed into one whose major activity was $CH_4$ formation. As in Bottle Sets 2 and 3, a high measurement of pH on Day 16 was indicative of a purging mishap on Day 12. This resulted in residual PCE measurements on Day 16. The amount of YE supplied to the system provided approximately an additional 44 microequivalents of reductant every 2 days in this bottle set. This could partially explain the accounting of products in excess of the amount of equivalents supplied to the system by the electron donor, MeOH.

## D. DISCUSSION

From the results of this experiment, it is evident that at the low level of PCE supplied to the cultures (9.1 mg/L), dechlorination of PCE was sustained only if higher ratios of MeOH:PCE were employed. However, even then, the production of ETH gradually decreased as an increasing fraction of the added PCE remained as VC after 2-day intervals. It seemed evident that the high-PCE culture cannot both sustain dechlorination of low levels of PCE and maintain consistently high ETH production, if MeOH is employed as an electron donor and methanogenesis is not inhibited. Bottle Set 1 indicated sustainable dechlorination and high HAc production, since the inhibition of the conversion of MeOH directly to $CH_4$, presumably due to the high level of PCE employed, allowed for the fermentation of the MeOH to HAc, thereby creating an intermediate pool of $H_2$ accessible to dechlorinating bacteria. However, in Bottle Sets 2, 3, and 4, the low level of PCE supplied did not inhibit the direct conversion of MeOH to $CH_4$, and acetogenesis and dechlorination became minor activities of the cultures as methanogenesis proliferated. It is apparent that if a low level of PCE is to be dechlorinated by the high-PCE culture, then the addition of an inhibitor of methanogenesis, but not dechlorination, might be desirable in order to prevent methanogenic bacteria from dominating within the culture. Alternatively, a nonmethanogenic source of $H_2$ might be usefully employed, lessening competition for the supplied electron donor. Still, however, methanogenic competition for $H_2$ must be reckoned with — a factor not addressed by these experiments.

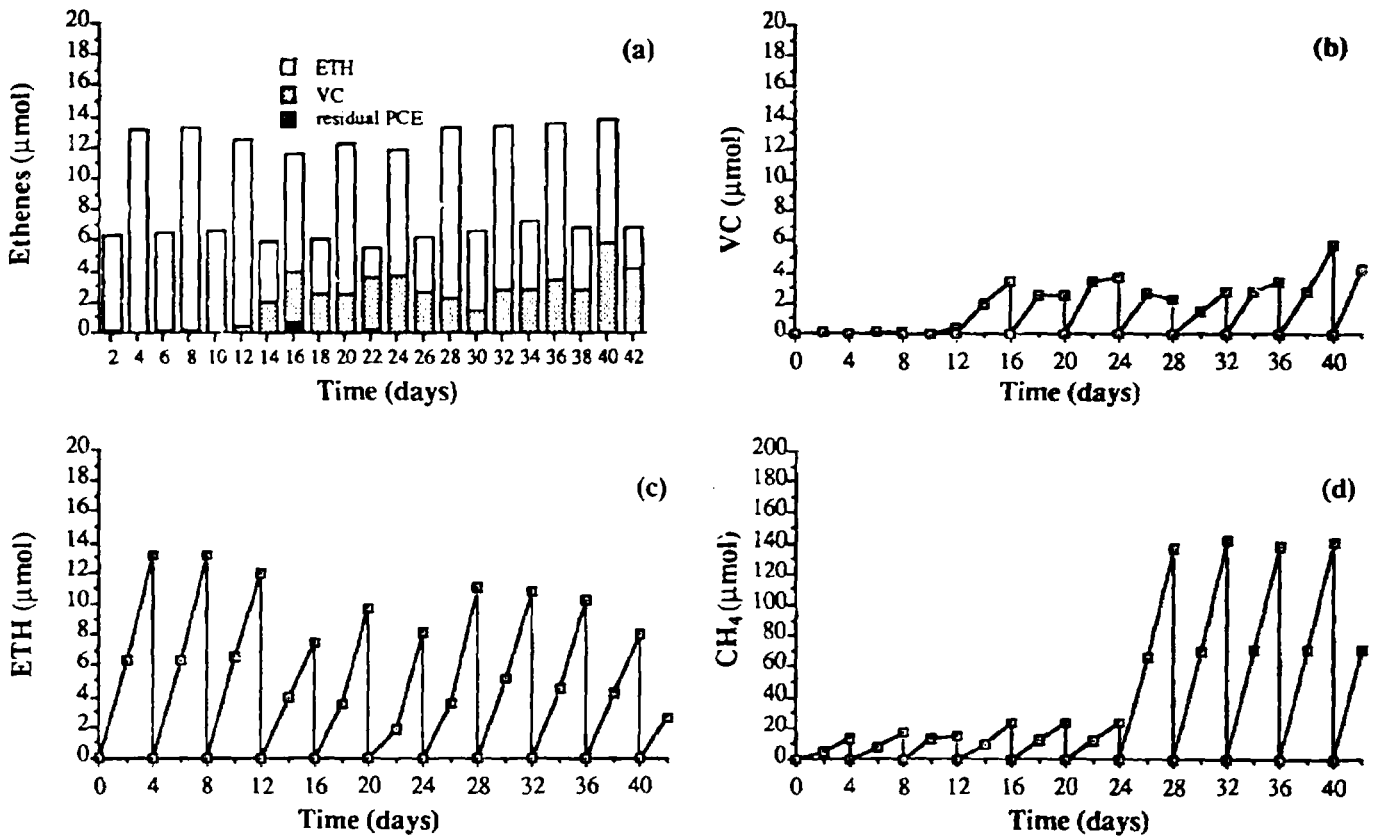



Figure 22. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) $CH_4$ Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 4A.

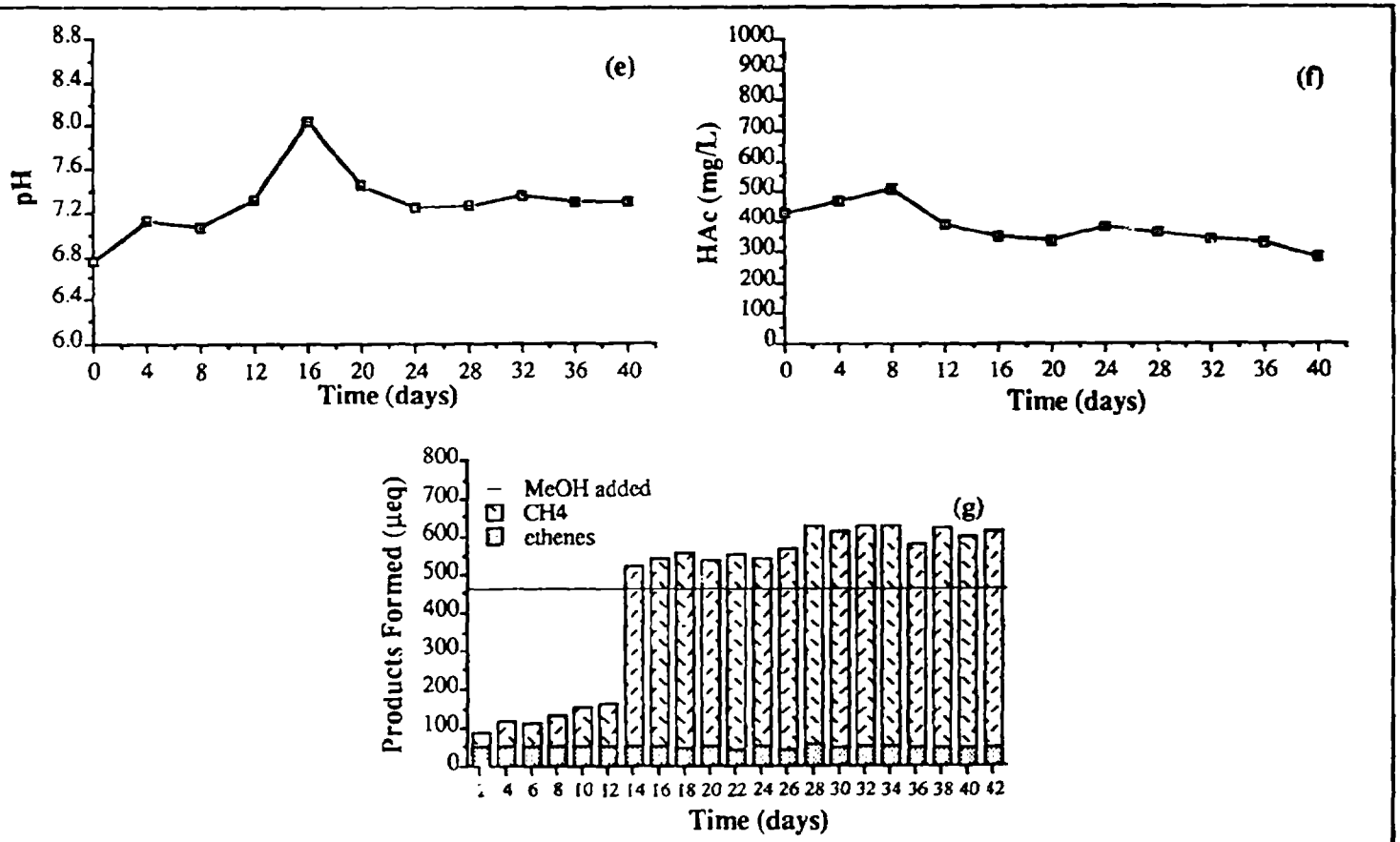



Figure 22. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) $CH_4$ Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 4A (Concluded).

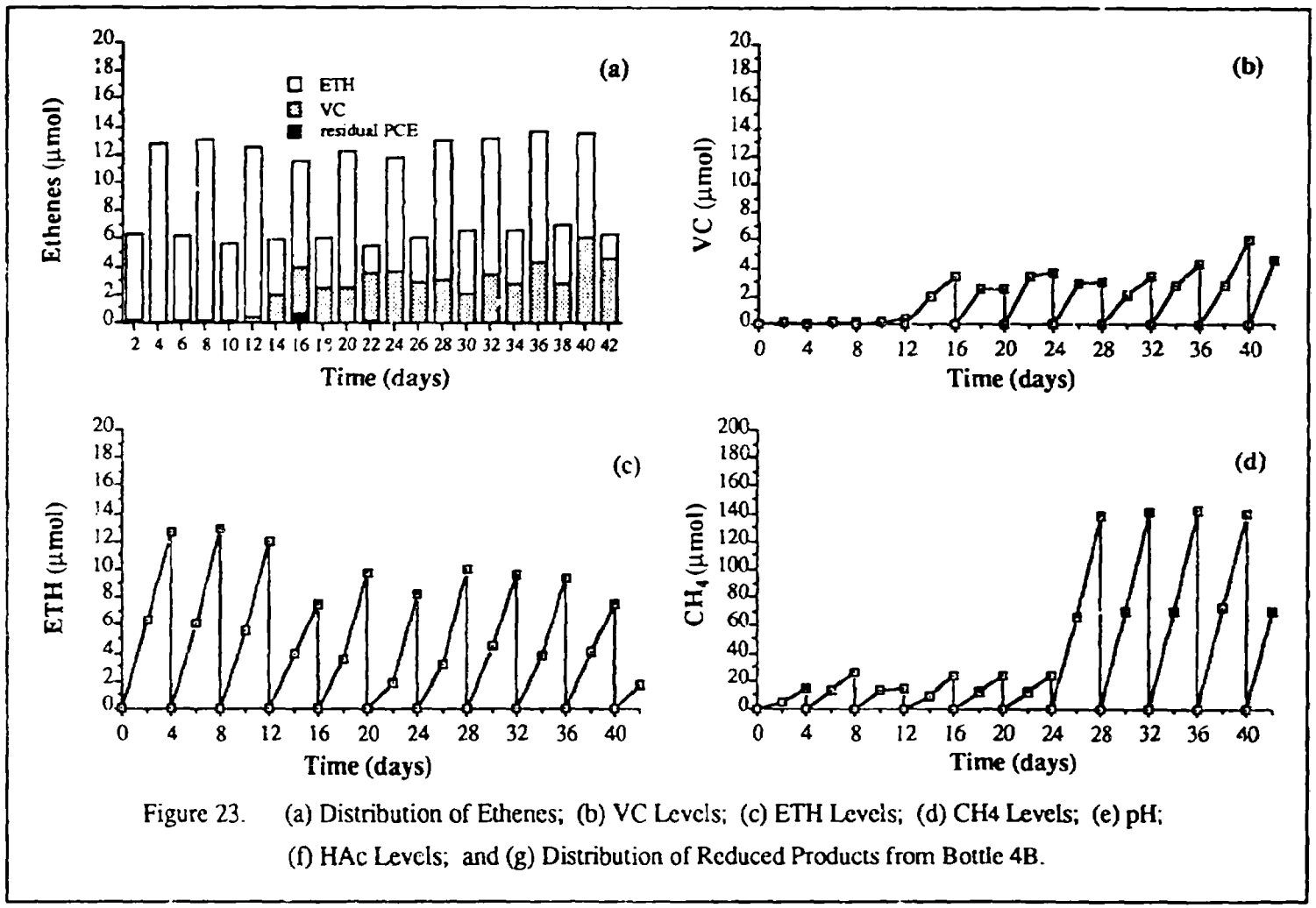



Figure 23. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 4B.

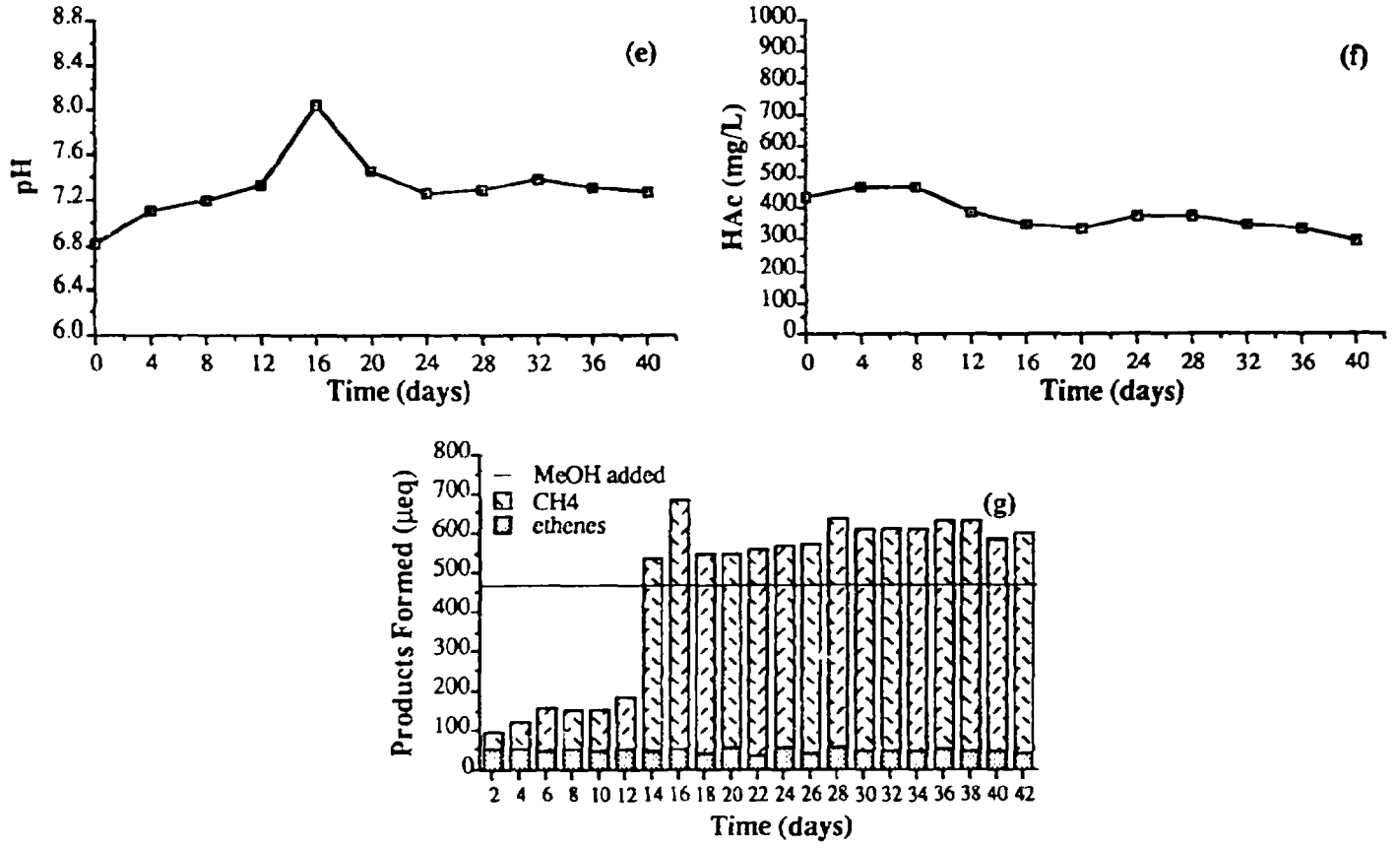



Figure 23. (a) Distribution of Ethenes; (b) VC Levels; (c) ETH Levels; (d) CH4 Levels; (e) pH; (f) HAc Levels; and (g) Distribution of Reduced Products from Bottle 4B (Concluded).

# SECTION VII

# CONTINUOUS-FLOW REACTOR STUDIES

## A. CONTEXT

Previous column studies conducted by DiStefano involved a laboratory scale, fixed-bed attached-growth column which was maintained in a plug-flow fashion (29). The conversion of PCE to VC and ETH was routinely observed. Although PCE was not detected after the first sampling port on the column, VC was routinely observed throughout the entire length of the column. DiStefano speculated that the presence of PCE may be necessary to stimulate dechlorination of VC to ETH, since VC was persistent in the downstream section of the column where PCE was not detected. It was suggested that a column reactor operated in a completely-mixed upward-flow fashion might facilitate better dechlorination since all of the organisms would be equally exposed to PCE and the electron donor. This reactor could be maintained as an AAFEB column reactor (42), wherein the biomass would be attached to the support medium, diatomaceous earth. The advantages of such a reactor are that relatively short HRTs and high mean cell residence times could be achieved since the biomass would be contained within the reactor and attached to the support medium.

## B. REACTOR DESCRIPTION

The AAFEB reactor system employed in these studies is depicted in Figure 24. The column body was constructed from 51-mm ID x 60-mm OD x 914-mm conical glass pipe (Corning Glass Works). All of the components of the column consisted of either stainless-steel, glass, or Teflon®, with five PTFE® O-rings. The top of the column incorporated a stainless-steel plate, bolted to the column by way of an aluminum flange, Teflon® gasket and a soft conical insert (Corning Glass Works). The top plate also contained a stainless-steel valve (Nupro Co.) and gas-sampling septum. Column gases only contacted the septum when the valve was opened for gas sampling via syringe and a 4.5-inch needle, which was long enough to penetrate through the septum and opened valve, directly into the column headspace. The bottom of the column consisted of a custom-machined, stainless-steel flanged cone, which was sealed and bolted to the column in a manner similar to that of the top plate previously described. All tubing and valves shown in Figure 24 were stainless-steel, with the exception of an 11-inch section of Viton® tubing (Cole-Parmer Co.; 0.8-mm ID), used in conjunction with the peristaltic feed-pump (Cole-Parmer Masterflex

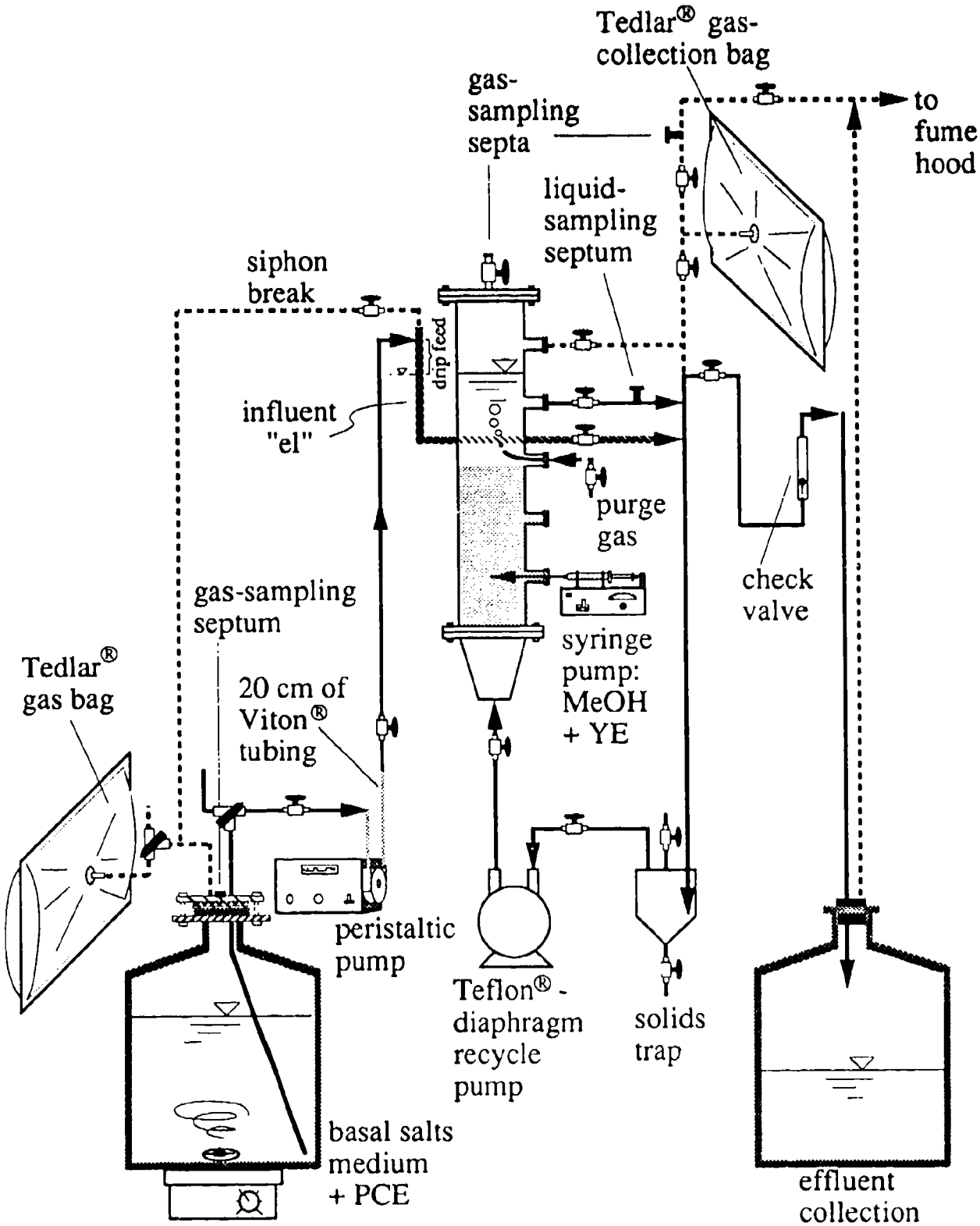



Figure 24. AAFEB Reactor System.

Model #7520-35), and short lengths of Viton® tubing which connected Tedlar® bag fittings to stainless-steel tubing. The peristaltic pump delivered influent basal medium containing dissolved PCE.

Five ports were located on the column, spaced every 152 mm (6 inches) from the base. Each port was constructed from a 14-mm ID x 22-mm OD threaded glass connector tube (Ace Glass Inc.), fused by a glassblower in such a way that it protruded approximately 48 mm from the outside wall of the column. Inside the bottom two connectors were custom-fabricated stainless-steel sampling/feeding tubes (2.0-mm ID x 12.7-mm OD x 102-mm) which extended into the center of the column, thereby minimizing wall effects during feed introduction from the syringe pump. The outside end of the sampling tubes were machined to fit an 11-mm Teflon®-faced rubber septum and aluminum crimp cap (Supelco, Inc.); the outside diameters of the sampling tubes were stepped down from 12.7 mm to 2.5 mm for the length of tubing extending from the wall of the column to the center of the column. Teflon® bushings and FETFE® O-rings (Ace Glass Inc.) secured the sampling tubes inside the connectors, and created a leak-tight seal.

The top three ports contained stainless-steel tubing which was secured with similar bushings and O-rings described above. The top port served to connect the column headspace with a Tedlar® 3.8-liter gas-collection bag (Cole-Parmer Co.); the second port from the top served as a conduit for combined effluent/recycle flow and included a septum for liquid sampling; the third port allowed for the introduction of anoxic $N_2/CO_2$ purge gas, which was necessary for column start-up and for purging of accumulated VC and ETH when the column was operated at high loading rates of PCE.

Dissolved PCE in an anaerobic basal-salts medium was delivered to the column via a peristaltic pump, as described previously. A 20.3-liter Tedlar® bag (Cole-Parmer Co.) was employed in conjunction with a 15-liter liquid feed reservoir (Fisher Scientific), to maintain a constant headspace volume in the feed reservoir at all times. The reservoir vessel was sealed with a Teflon®-lined stainless-steel plate which was secured to the mouth of the reservoir with an aluminum flange. A one-inch section of Viton® tubing joined the gas bag with stainless-steel tubing, which protruded through the top plate and into the headspace of the reservoir. When the reservoir was refilled (approximately twice monthly) with fresh PCE and basal medium, the reservoir headspace was displaced into the gas bag. As the feed reservoir liquid volume dropped slowly over the 2-week feeding period, the PCE-laden headspace was transferred back into the bottle. This system was intended to prevent loss of PCE concentration in the feed reservoir via partitioning to an otherwise expanding headspace. The reservoir system therefore attained a steady-state condition in which the headspace concentration, $C_g$, remained relatively constant ( = $H_c C_w$, in which $C_w$ is the steady-state aqueous concentration of PCE in the feed, and $H_c$ is Henry's constant for PCE).

MeOH was dissolved in water at a 1:30 MeOH:$H_2O$ ratio (mass basis) and delivered to the reactor via a syringe infusion pump (Harvard Apparatus, Model #22). The 5-mL syringe used for delivery was replenished with the MeOH/water mixture each week. A 24-inch length of Neoprene® tubing was employed to deliver the MeOH/water mixture from the syringe to a 4.5-inch needle which protruded from the bottom port into the center of the reactor body.

The bed medium consisted of diatomaceous earth particles (Celite 560, Monarch Chemical) of a 100-μm average diameter. The unexpanded volume originally measured approximately 500 mL, but because of an accumulation of solids within the bed, the unexpanded bed volume measured 600 mL at the end of the column study. The expanded bed volume measured 700 mL throughout the operation of the column. The expansion of the bed varied, therefore, from approximately 17 percent to 30 percent during the course of the study. The recycle pump which caused the bed expansion incorporated a Teflon®-diaphram pump head (Cole-Parmer Masterflex Model #7520-25). A solids trap, located upstream from the diaphragm pump, prevented solids from entering the pump and causing failure of the check-valves and diaphragm. The trap consisted of a glass vessel capped with a stainless-steel plate which was secured similarly to the plates on the top and bottom of the column. The recycle fluid entered the trap through the top plate, and exited the trap via a protruding port similar to the top three ports of the column body. The recycle flow then passed through the diaphragm pump and directly into the column.

The basal medium/PCE mixture was delivered to the column through an influent "el" and into the recycle flow. This "el" was connected to the reactor's recycle line at a point which was located a few inches below the point of exit of the recycle flow from the supernatant zone of the reactor. Since the recycle flow was much greater than the effluent flow, the basal medium/PCE feed introduced at this point in the recycle was completely swept downward, with no short-circuiting to the effluent. The headspace of the drip-feed zone was connected to the headspace of the feed reservoir, providing a siphon break; this design ensured that the level of influent fluid in the "el" equaled that of the supernatant in the reactor, regardless of flow or loss of peristaltic seal.

Effluent passed through a gas trap, then past a check valve consisting of a section of glass tubing which was beveled on the inside, thereby allowing a Teflon® bead ($1/4$-inch) to act as a seal against the diffusion of oxygen into the system. Effluent then flowed into a collection reservoir, which was vented to a fume hood. Constant pressure was initially maintained in the reactor since the headspace was connected to the gas collection bag; however, the gas bag was later isolated from the system since it was suspected that oxygen was diffusing into the reactor through the bag. Reactor conduits consisted of either $1/2$-, $1/4$-, or $1/8$-inch stainless steel tubing, connected with Swagelok® fittings, except otherwise noted, and various stainless-steel valves (Nupro Co., Whitey Co.) were located throughout the reactor body, as indicated in Figure 24, for maintenance and operation purposes.

## C. AAFEB LIQUID AND GASEOUS VOLUME MEASUREMENTS

The liquid volume of the column reactor was determined by a measurement (via atomic absorption spectroscopy with air/acetylene flame) of potassium concentrations before and after a known amount of potassium was injected into the column. The column initially contained a low concentration of potassium since the test was performed when the reactor was already inoculated with culture medium containing basal salts medium which included approximately 50 mg/L $K^+$. A highly concentrated slurry of potassium chloride (KCl) was injected into the reactor after an initial potassium concentration measurement was made. The volume of this concentrated slurry of KCl resulted in a concentration in the reactor of potassium of approximately one order of magnitude greater than the initial potassium concentration. With knowledge of the difference in concentration of potassium in the reactor — that is, initial minus final concentration — along with knowledge of the amount of potassium injected into the column, the liquid volume of the reactor was calculated. The gaseous volume of the reactor was simply measured via a displacement method, in which the reactor headspace was filled with basal medium (anoxically, since the reactor was inoculated at this time) with the gas bag isolated from the system, and then allowed to drain to the normal liquid equilibrium level. The effluent which drained from the filled reactor was measured and therefore the gaseous volume was determined. Results of these determinations were as follows: liquid volume = 1.56 liters; gas volume = 0.49 liters.

## D. AAFEB SYSTEM TESTS

The top graph of Figure 25 indicates the results of a PCE-loss test from the reactor system while the reactor, containing only PCE and water, was operated in recycle mode. Initially, an amount of PCE was added to the reactor which resulted in an initial concentration of approximately 1.6 mg/L PCE. Measurements of the aqueous concentration of PCE over the next 8 days indicated an average loss of PCE of 5.4 percent per day.

The middle graph of Figure 25 shows the results of PCE loss tests conducted on the feed-reservoir system. PCE (10 mg/L nominal concentration) was added to 15 liters of distilled water in the reservoir. After approximately one week of equilibration between the liquid and gaseous phases of the feed-reservoir system, a headspace analysis indicated that an aqueous concentration of 6.5 mg/L was achieved. The subsequent change in this concentration was monitored over a 2-week period. During the initial 7 days, no liquid was pumped from the reservoir; during the final 7 days, the peristaltic pump began to remove approximately 1 liter per day from the reservoir, forcing gas-volume from the Tedlar® bag back into the glass carboy. The results shown in Figure 25 indicate no significant difference in loss rate between the initial 7 days (in which no pumping occurred) and the final 7 days (in which pumping occurred). In addition, the results indicated

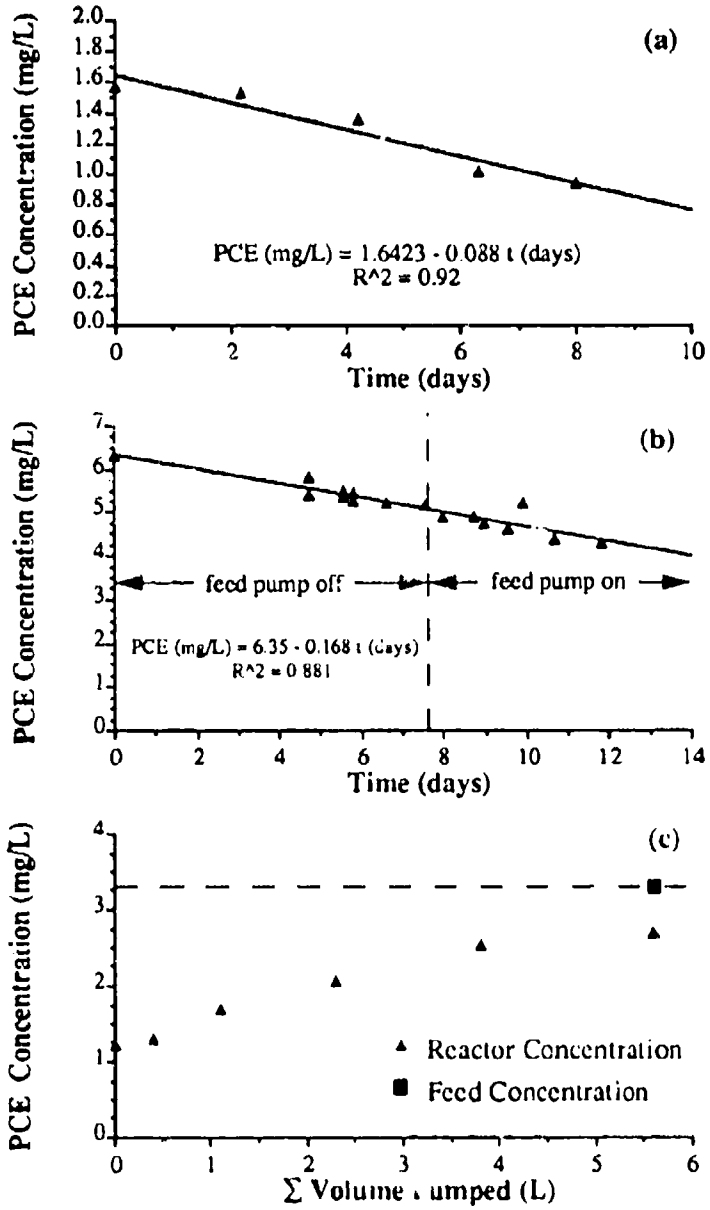



Figure 25. System Tests Conducted on AAFEB system: (a) PCE Loss From Column Reactor; (b) PCE Loss From Feed Reservoir; and (c) PCE Reactor Test With Pumped Influent.

a loss rate of less than 2.7 percent per day, which was deemed acceptable for a 14-day planned operation interval between reservoir replenishments.

The final graph of Figure 25 illustrates the results obtained from a third system test in which PCE losses from the column and peristaltic pump were evaluated. The aqueous PCE concentration in the reactor was calculated from column headspace analysis by invoking Henry's constant for PCE. At time zero, the reactor concentration was 1.2 mg/L PCE (remaining from some previous loss studies). The peristaltic pump was then started, pumping approximately 1 L/d of distilled water from the feed reservoir which contained an aqueous concentration of PCE of 3.4 mg/L. The recycle (diaphragm) pump was operated throughout the study. Over a period of several days, the aqueous concentration of PCE was monitored from headspace samples. Figure 25 indicates that the reactor concentration was building towards the influent concentration, suggesting an acceptably low rate of PCE loss in both the peristaltic pump and reactor system.

## E. INOCULATION AND PERFORMANCE OF THE AAFEB REACTOR

Once the design and operation of the reactor system were deemed acceptable, the reactor was inoculated with approximately 500 mL of the MeOH-fed high-PCE enrichment culture contained within the 6-liter, stirred reactor, described in Section III. Initially, the reactor system was operated in batch-fed recycle mode at 35°C so that the bacteria would become acclimated to the column environment. During this time, the recycle pump ran continuously to expand the bed; PCE, MeOH and YE were fed in concentrations similar to those supplied to the high-PCE/MeOH inoculum source. Every 2 days, the reactor supernatant zone was purged for 30 minutes with an anoxic gaseous mixture of $N_2/CO_2$ to remove VC and ETH; MeOH and neat PCE were injected with separate syringes to achieve a 2:1 stoichiometric MeOH:PCE ratio on an electron equivalent basis. YE was delivered by syringe to achieve a concentration of 20 mg/L in the reactor. During this batch-fed mode, all feed was delivered through 4.5-inch needles and injected through the lowest port of the column body, into the center of the expanded bed.

After a period of instability and reactor modifications, the system began to dechlorinate quite well; that is, nearly all of the injected PCE was converted to VC and ETH, with little PCE left. Measurements of gaseous and aqueous concentrations of volatile compounds were made from headspace analyses with knowledge of Henry's constants for each compound (43), and the liquid and gaseous volumes of the reactor. After 3 weeks of batch-fed operation, the reactor supernatant was displaced with fresh basal medium which was delivered via the peristaltic pump from the feed reservoir. This introduction of fresh basal medium was not performed continuously, but rather at relatively high rates for short periods of time every other day, so as to simulate as closely as possible the operation of the 6-liter inoculum-source reactor.

After 2 weeks of operation in this semicontinuous mode, the reactor was prepared to commence in a continuous-flow mode. Initially, a MeOH, YE, and water mixture was delivered to the reactor with a syringe pump; however, because growth was evident in the feed line, indicating probable consumption of MeOH and spoilage of YE, this method was abandoned. Instead, a mixture of only MeOH and water was delivered with a syringe infusion pump; thereafter, YE was either dissolved in the basal medium/PCE reservoir or delivered directly to the reactor by syringe every 2 days. Basal medium containing dissolved PCE at 100 mg/L was delivered to the reactor at a rate of 0.4 L/day, resulting in a PCE loading rate of 40 mg/day. The resulting ratio of MeOH:PCE supplied to the system was 2:1, on an electron equivalent basis. The HRT, which was calculated using the volume of the expanded bed and the PCE pumping rate, was therefore 1.8 days.

Once continuous flow commenced, the reactor was operated for 200 days. Four stages of column reactor performance during this period of time were analyzed; the stages are summarized as follows. Initially, the culture failed to sustain dechlorination when the column was operated continuously, and an apparent channeling of reducing equivalents toward acetogenesis was observed. Second, as dechlorination worsened, a shift toward methanogenesis became apparent while continuous flow resumed in the column reactor system. Third, while the column was operated in recycle mode (to attempt recovery), the application of a methanogenic-inhibiting concentration of ETH appeared to spur HAc production. Finally, during a fourth stage of column performance, methanogenesis continued to be inhibited, but dechlorination worsened as the column reactor was operated once more in a continuous-flow fashion with a high background concentration of ETH.

Figure 26, depicts reactor performance for the first 75 days of operation. Values of aqueous concentrations of influent PCE and of PCE, VC and ETH in the effluent are plotted against time. Increasing concentrations of effluent PCE and corresponding declining concentrations of VC and ETH in the effluent indicate the difficulty experienced by the culture while the column was operated in a continuous-flow mode. A mishap on Day 38 resulted in the infiltration of air into the system; the reactor was subsequently purged with an anoxic gaseous mixture of $N_2/CO_2$, causing a decrease of volatiles in the effluent, and a lack of agreement between the influent PCE concentration and the sum of the concentrations of ethenes in the effluent. The reactor was reinoculated on Day 53, yet through Day 75, the PCE in the effluent continued to rise. During this time, VC production increased slightly but ETH concentrations remained low in the effluent. Since it became evident that reducing equivalents were not being utilized for methanogenesis or dechlorination to any significant degree, liquid samples for measurements of HAc were removed from the reactor, beginning on Day 28.

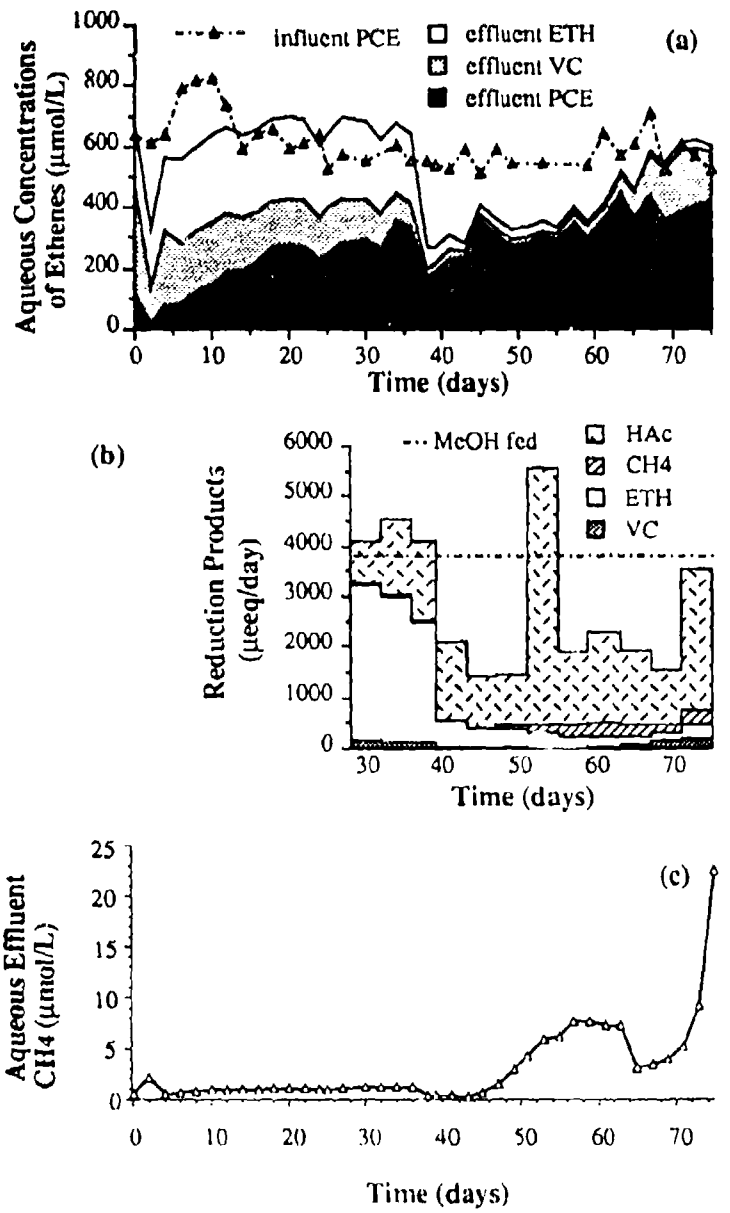



Figure 26. (a) Influent and Effluent Aqueous Concentrations of Ethenes; (b) Rate of Reduction Product Formation; and (c) Effluent Aqueous $CH_4$ Concentration During Days 0-75 of AAFEB Operation in Continuous-Flow Mode.

The second plot (i.e., rate of product formation versus time) in Figure 26 indicates that acetogenesis accounted for a major fraction of the consumption of MeOH during the first 75 days of operation. Over defined time intervals, product formation rates were computed by noting average effluent concentrations *and* the change in product inventory within the gas and liquid volumes of the reactor system. Again, the imbalance between the rate of product formation and MeOH delivery after Day 38 was probably due to the purging of the reactor on this day. The rate of MeOH supplied to the reactor, represented in this plot and in subsequent figures, is a *theoretical* rate; MeOH was not measured in the influent, and the rate plotted in the figures was calculated from the rate at which the syringe pump was operated. The apparent surge of HAc on Day 53 was probably due to the reinoculation of the column, since the inoculum from the 6-liter high-PCE reactor contained a high concentration of HAc. Though the second plot of Figure 26 suggests that methanogenesis was not a major activity during this initial 75-day period of operation, the third plot of Figure 26 indicates that methanogenic activity was definitely on the increase.

A second stage of column performance from Days 92-119 is illustrated in Figure 27. During this period of continuous flow, PCE in the effluent remained high, and production of VC and ETH worsened over time. The rate of reduced product formation over time indicates that methanogenesis accounted for an increasing fraction of the donor supplied during this period, and this rise in methanogenesis is confirmed by the plot of increasing $CH_4$ concentration in the effluent over time, possibly due to the absence of a high background concentration of ETH [a known inhibitor of methanogens (44)]. The equivalents released from endogenous sources and consumption of organic material in the YE supplied to the system might partially account for the imbalance in the higher rate of reduced product formation in comparison to the rate of MeOH fed to the reactor. Alternatively, a proliferation of HAc-consuming methanogenic bacteria could have been responsible for the high $CH_4$ levels measured within this period. The reactor was purged on Day 95 in order to clear a clogged portion of the recycle tubing; the observed loss of volatiles was due to this purging.

Figure 28 illustrates the performance of the column reactor while it was operated in recycle mode during Days 119-145. Additions of PCE were not made during this time, but MeOH was continuously fed as before, and YE was supplied every 2 days. A high level of ETH was maintained in the column headspace during this time, as noted in the Figure, and the resulting inhibition of methanogenesis was evident by the declining rate of $CH_4$ production within the column. Inhibition of methanogenesis was desired since the channeling of reducing equivalents toward methanogenesis during the previous period of continuous flow resulted in a decreasing fraction of MeOH available for dechlorination. A plot of the accumulation of reduced products

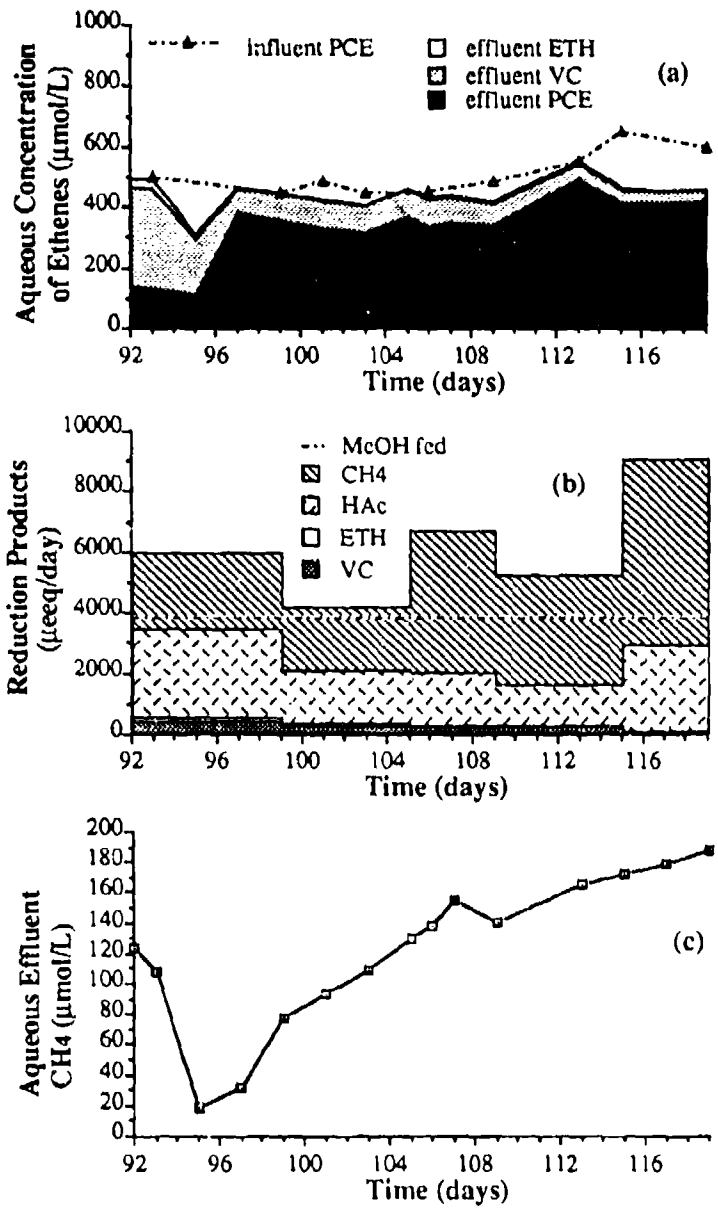



Figure 27. (a) Influent and Effluent Aqueous Concentrations of Ethenes; (b) Rate of Reduction Product Formation Over Time; and (c) Effluent Aqueous CH4 Concentration During Days 92-119 of AAFEB Operation in Continuous-Flow Mode.

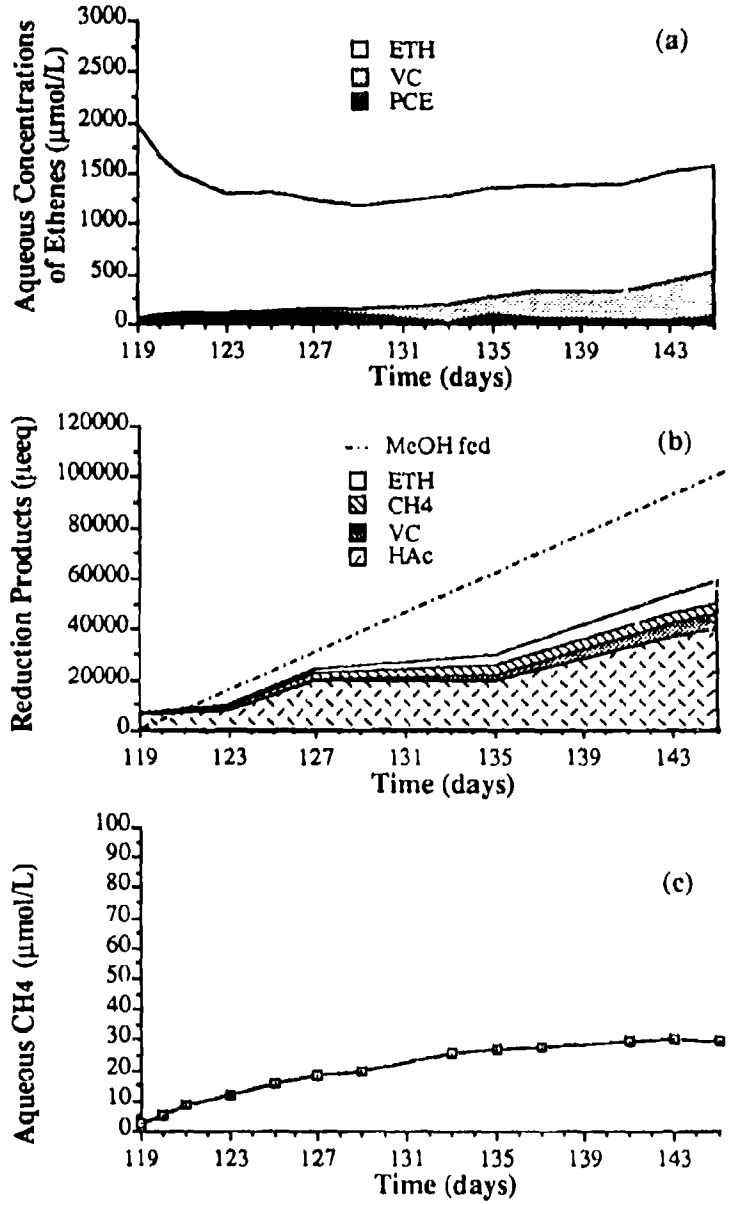



Figure 28. (a) Aqueous Concentrations of Ethenes; (b) Reduction Product Formation; and (c) Inhibited Methanogenesis During Days 119-145 of AAFEB Operation in Recycle Mode With a High Background Concentration of ETH.

during this time indicates that acetogenic bacteria consumed a major fraction of the MeOH supplied to the system.

The lack of agreement between the theoretical amount of equivalents supplied to the system and the sum of reduced products could be due to losses in the delivery of MeOH, loss of MeOH to the effluent, or consumption of MeOH (or $H_2$) by organisms other than methanogens, acetogens or dechlorinators, such as sulfate-reducing bacteria. It was observed that the basal medium within the feed-reservoir appeared to lighten in color after 2-week periods, indicating that the sulfide within the medium was oxidized to sulfate or thiosulfate by an unidentified oxidizing agent, thereby possibly creating an electron acceptor for sulfate-reducing bacteria. Since sulfate-reducing bacteria have been identified within the high-PCE culture which served as the inoculum for the column reactor (40), it is possible that these sulfate-reducers consumed a portion of the $H_2$ formed. The medium contained within the column reactor might have experienced this very transformation, although no experimental tests were undertaken to verify this hypothesis.

The column reactor was operated in continuous-flow mode with a high background concentration of ETH from Day 169 to the conclusion of the column analysis on Day 200. Basal medium containing dissolved PCE was delivered to the reactor at the same rate as before, at 0.4 L/day. Figure 29 indicates that although methanogenesis continued to be inhibited by the high level of ETH employed, dechlorination worsened with time.

The results from the column study seem to indicate that although the high-PCE culture was able to sustain dechlorination of high levels of PCE while maintained in the column reactor during semicontinuous operation, once truly continuous flow commenced, dechlorination performance worsened over time, even when methanogenesis was inhibited. Acetogenic bacteria, however, did not appear to be adversely affected by the continuous-flow operation; methanogenic bacteria seemed to proliferate during continuous-flow only in the absence of high background concentrations of ETH. It is plausible that the dechlorinating population of bacteria experienced an inability to adhere effectively to the support medium, whereas other populations of bacteria might have adhered to the diatomite without difficulty. Shear created by the expansion of the bed might have caused "washout" of the dechlorinating bacteria, thereby decreasing the population of these bacteria, and resulting in decreasing dechlorination over time.

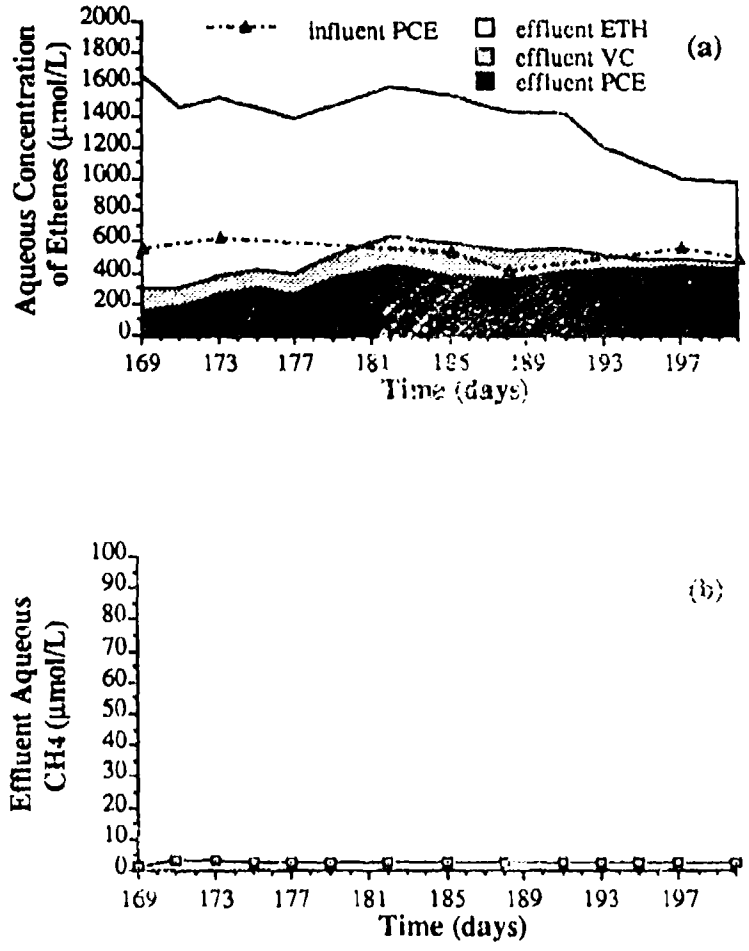



Figure 29. (a) Influent and Effluent Concentrations of Ethenes; and (b) Effluent Aqueous $CH_4$ Concentration During Days 169-200 of AAFEB Operation in Continuous-Flow Mode with a High Background Concentration of ETH.

# SECTION VIII

# CONCLUSIONS AND RECOMMENDATIONS

Results from these current studies strongly suggest that $H_2$ is the electron donor directly used for the reductive dechlorination of PCE to ETH by organisms in our MeOH/PCE enrichment culture. A model (Figure 15) is presented, depicting hypothetical roles for methanogens and acetogens as producers of an intermediate pool of $H_2$ in the culture. If borne out by subsequent studies, our hypothesis that $H_2$ is the actual electron donor for PCE dechlorination has practical consequences for bioremediation. It explains why a wide variety of electron donors has been observed to support PCE dechlorination in mixed-culture systems. All likely produce at least a small pool of $H_2$; and a small pool would be sufficient to transform ppb-levels of PCE. For bioremediation of relatively high PCE levels, electron donors should be selected which cause production of a large $H_2$ pool — or methods may be explored which would employ $H_2$ directly. One caveat is the apparent nutritional dependency we observed of $H_2$-using dechlorinators in our culture upon other organisms. If dechlorinating organisms in other systems have the same or similar nutritional requirements, the required nutritional factor(s) must be identified and abiotically supplied, or it may be impossible to exclusively target electron donor solely to the dechlorinators.

Our high-PCE/MeOH culture makes relatively efficient use of MeOH in dechlorination — sustained dechlorination was achieved at MeOH/PCE ratios on the order of 2:1 (eq/eq). This efficiency was not observed in our initial studies with a low-PCE/MeOH enrichment culture (1), where rapid, uninhibited methanogenesis out-competed acetogenesis for MeOH. The interspecies $H_2$ pool developed by methanogenic use of MeOH is thought to be considerably lower than that developed by acetogenic use of MeOH. At low PCE levels (i.e., ca. 1-10 mg/L), where methanogenesis from MeOH is uninhibited, results from our studies indicate a required MeOH/PCE (eq/eq) ratio approaching 100:1. Furthermore, methanogenesis from MeOH is so rapid that in many instances, MeOH is depleted before dechlorination is complete.

The efficiency of our high-PCE/MeOH culture apparently results from the inhibition of MeOH-using methanogens by high PCE (or dechlorination product) concentrations, thus lessening competition for MeOH. When non-inhibitory concentrations (i.e., 9 mg/L) of PCE are employed with the high-PCE/MeOH culture, methanogenic activity rises and consumes an ever-increasing fraction of MeOH equivalents (Figures 18-21), necessitating ever-greater additions of MeOH to allow sustenance of dechlorination. However, this tactic is a spiral to failure, as ever-greater additions of MeOH support ever-larger methanogenic populations. This same phenomenon may have contributed to the failure of our continuous-flow bioreactors (Figures 26 and 27).

In essence, we have observed that PCE dechlorination is relatively easily accomplished at extremes of concentration: At very high concentrations (> 20 mg/L), PCE (or its dechlorination products, such as ETH) inhibits competition by methanogens for MeOH and $H_2$; at very low concentrations (ppb levels), competition causes little concern because only a minute fraction of the donor is required for dechlorination, and this requirement is easily met. But intermediate PCE levels are problematic, as are high influent levels to continuous-flow reactor configurations in which the reactor biomass is exposed only to low, *effluent* PCE levels. Here, the dechlorination requirement for reducing equivalents is high, and methanogenic competition is intolerable.

One potential solution to the dilemma posed by methanogenic competition would be the use of nonmethanogenic substrates that can act as sources of $H_2$. Ethanol, lactate, and butyrate would be good substrates for investigation; $H_2$ is a direct product of their fermentation to HAc. Since they are not direct methanogenic substrates (ethanol can be used by some methanogens but poorly and not as a direct $CH_4$ precursor), they may prove to be more efficient suppliers of reducing equivalents for dechlorination, and they are less toxic to humans than MeOH. It remains to be seen with what efficiency our culture might operate on them. Of particular interest is whether such alternative substrates might eliminate nutrient deficiencies observed when our high-PCE culture was fed $H_2$ directly.

Alternative, nonmethanogenic substrates might eliminate direct methanogenic competition for the supplied form of the reductant; however, methanogenic competition for the resulting $H_2$ would still have to be reckoned with. Possible differences in $H_2$-use thresholds between methanogens and dechlorinators may be exploited to advantage in this context. Possible differences in environmental preferences (e.g., pH) might also be advantageously exploited. Thus, the $H_2$-consumption kinetics of methanogens and dechlorinators should be investigated — including studies of the nutritional and environmental requirements of the organisms. In this manner, strategies may be developed to make optimal use of supplied reductant while minimizing undesirable competing reactions.

Results from studies with low-PCE/MeOH enrichment cultures (Section IV) suggest that the presence of PCE (and /or TCE, DCEs) is (are) required to sustain VC dechlorination. Examination of Figure 3 indicates that appreciable ETH production did not occur until VC reached 2.5 µmol, a trend which was observed numerous times in other PCE-fed bottles. This lends support to the hypothesis that PCE *and* high levels of VC are required before ETH production is substantial. The presence of PCE may be necessary to stimulate production of a non-specific enzyme capable of mediating dechlorination. Recall that when PCE was withheld, and then reintroduced, a lag in dechlorination was noticed (Figure 6). Such a lag may mean that the organism is dechlorinating PCE for a benefit. Indeed, the observation that PCE dechlorination seems to be inducible, and therefore confers a benefit to the mediating organism, was noted

previously (30). The comparatively rapid conversion of PCE to VC also suggests metabolism; whereas the relatively slow conversion of VC to ETH suggests this last step might be a cometabolism (i.e., a serendipitous, coincidental transformation.) It is not obvious whether both transformations are mediated by the same enzyme system — or even by the same organism. These determinations await the outcome of more fundamental, microbial studies.

The results from our column studies seem to indicate that although the high-PCE culture was able to sustain dechlorination of high levels of PCE while maintained in the column reactor during semi-continuous operation, once truly continuous flow commenced, dechlorination performance worsened over time. Failure may have resulted from methanogenic competition (discussed above). However, resurrection of performance was not achieved, even when methanogenesis was inhibited by high levels of supplied ETH. Shear created by the expansion of the bed might have caused "washout" of the dechlorinating bacteria, thereby decreasing the population of these bacteria, and resulting in decreasing dechlorination over time. (However, it must be noted that methanogens and acetogens experienced no apparent difficulty maintaining their populations in the reactor system.) In addition to exploring alternative electron donors, investigators should explore alternative reactor support matrices and alternative configurations which may offer superior organism retention.

Development of successful anaerobic biological treatment technologies — and development of groundwater contaminant transport models which accurately incorporate anaerobic transformation mechanisms — demand that the requirements and capabilities of the microorganisms responsible for observed biodegradation be defined, as well as their interactions with symbiotic and competing microbes. Our recent failure to achieve sustained dechlorination at "moderate" (but not inhibitory) levels of PCE using MeOH demonstrates the folly of conducting "black-box" bioreactor studies. While much can be learned from empirical studies, they can be likened to the studies of wine-making before Pasteur. If we had not discovered the importance of interspecies $H_2$ transfer to the dechlorination process — and discovered it through relatively fundamental, bench-scale studies — we would have no clue as to the reason for failure (methanogenic competition), nor would we have reasonable, *fundamentally based* remedies to propose (i.e., alternative electron donors). Insights from our results may be useful to researchers designing bioremediation strategies and modeling the fate of these compounds *in situ*. An understanding of the microbiology of reductive dechlorination of PCE can only enhance our ability to understand and control the processes that may remove it and other chlorinated ethenes from the environment.

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